THE FUNDAMENTAL PROPERTIES OF THE FIBROBLAST AND THE MACROPHAGE.

II. The Macrophage.

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Plates 8 and 9.

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The purpose of the experiments described in this article was to elucidate the relations of the macrophage[1] and the monocyte,[2] and to define these cells by their essential biological properties.

Monocyte and Macrophage as Functional Variations of a Single Type.

The macrophages which migrated from fragments of connective tissue, organs, tumors, etc., of adult animals did not differ markedly from the blood monocytes which had been cultivated *in vitro* for a few days. This fact rendered it probable that both cells merely represent metabolic states of a single type, and that their structure can be modified at will by certain changes in the composition of the medium. Two convergent methods were used. The first one consisted of placing macrophages derived from the subcutaneous connective tissue and monocytes obtained from the blood in media of identical composition, and ascertaining the structural changes occurring after a few days. If monocytes and macrophages merely express different metabolic states of a single cell, they must become identical in appearance when living under the same conditions. By the second method, monocytes and macrophages were studied in media of different composition in

[1] Synonyms: round rhagiocrin cells of Renaut; polyblasts of Maximow; clasmatocytes of Ranvier; endothelial leucocytes of Mallory; adventitial cells of Marchand; pyrrol cells of Goldmann.

[2] Synonyms: large mononuclear leucocytes of Ehrlich; endothelial leucocytes of Mallory; blood histiocytes of Aschoff.

order to determine whether metabolic changes can modify their cytoplasmic structures.

In a first series of experiments, fragments of leucocytic films and flaps of subcutaneous connective tissue from adult chickens were cultivated in a medium composed of plasma, Tyrode solution, and embryonic juice. After a period varying from 2 hours to a number of days, fragments of the colonies were removed from the flasks, transferred to cover-glasses, and stained with neutral red and Janus green. Camera lucida drawings were made at a magnification of 1600 diameters.

Experiment 1. Monocytes Freshly Removed from the Blood.—Culture 3002-A2, Jan. 14, 1926. Fragments of leucocytic films placed on cover-glasses in a hanging drop of plasma and embryo juice. After 2 hours, preparation stained; camera lucida drawings. A few monocytes are seen among many polymorphonuclear leucocytes. Length of cells, 10 to 15μ; active pseudopods; approximately 10 small neutral red vesicles about 1μ in diameter, containing 1 or 2 small granules; vesicles grouped around the centriole or scattered through the cytoplasm.

Experiment 2. Blood Monocytes after 2, 24, 48, 72, and 96 Hours Cultivation.—Culture 8740-D, Apr. 19. Fragments of leucocytic film cultivated on cover-glasses in equal parts of plasma and embryo juice. After 2 hours, preparation stained; camera lucida drawings. Large number of polymorphonuclear leucocytes (Fig. 1) migrating into the medium among a few red blood corpuscles (Fig. 2); no lymphocytes; some monocytes, all of identical appearance; cells 10 to 15μ long; 8 or 10 neutral red vesicles about 1μ in diameter, radially arranged around the centriole in a few cases; mitochondria as faintly stained dots; no fat globules (Fig. 3). Apr. 20, stained; camera lucida drawings. Active polymorphonuclear leucocytes, unchanged in size or appearance; a few lymphocytes. Without exception, all the monocytes have increased in size, being 30 to 40μ in length; no change in the mode of locomotion and in the general appearance of the cells. The neutral red vesicles are more numerous, from 0.25 to 1.5μ in diameter, and contain granules; no rosette disposition; mitochondria more deeply stained, assuming the shape of rods (Fig. 5). Apr. 21, stained; camera lucida drawings. Active polymorphonuclear leucocytes, unchanged in size or appearance; a few lymphocytes. All the monocytes have increased in size, being 40 to 45μ in length; no change in the mode of locomotion and in the general appearance of the cells; no rosette disposition of the vesicles. They are numerous, 0.5 to 2μ in diameter, and deeply stained. A number of the cells show partially digested polymorphonuclear leucocytes; mitochondria as round and rod-shaped granules, deeply stained (Fig. 6). Apr. 22, stained; camera lucida drawings. Decrease in the number of polymorphonuclear leucocytes. They are unchanged in size or appearance; no lymphocytes. Many of the monocytes are slender, 50 to 75μ in length; no change in the mode of locomo-

tion. The vesicles are very numerous, 0.5 to 2μ in diameter, and fill the trophoplasm. In some cases, cells show ingested red blood corpuscles and polymorphonuclear leucocytes; mitochondria as deeply stained granules (Fig. 7). Apr. 23, stained; camera lucida drawings. Very few active polymorphonuclear leucocytes. They are unchanged in size or appearance; no lymphocytes. Most of the monocytes are slender, 55 to 80μ in length. The undulating membrane has disappeared along the sides of the cells. The vesicles are 0.25 to 1μ in diameter, less numerous, and not as deeply stained as in the cells observed after 3 days incubation; no evidence of ingested cells. Mitochondria as deeply stained granules (Fig. 8).

Experiment 3. Blood Monocytes after 2 and 24 Hours Cultivation.—Culture 8741-D, Apr. 20. Fragments of leucocytic film cultivated on cover-glasses in equal parts of plasma and embryo juice. After 2 hours, preparation stained; camera lucida drawings. Normal polymorphonuclear leucocytes; all monocytes identical in appearance; cells 10 to 15μ long; 8 or 10 neutral red vesicles, showing the rosette arrangement in some cases; mitochondria as faintly stained dots; no fat globules; cells identical with those of Fig. 4. Apr. 21, stained; camera lucida drawings. Active polymorphonuclear leucocytes; a few lymphocytes. Every visible monocyte has increased in size, being 30 to 40μ in length; same mode of locomotion and general appearance; rod-shaped mitochondria. The neutral red vesicles are larger and contain dark granules; not grouped around the centriole; cells identical with those of Fig. 5.

Experiment 4. Blood Monocytes Cultivated for 7 Days.—Culture 3020-A2, Jan. 21. Fragments of leucocytic film cultivated in a flask containing 0.5 cc. plasma, 1 cc. Tyrode solution, and 0.5 cc. embryo juice. After coagulation, 0.5 cc. serum and 0.5 cc. embryo juice added to medium. Jan. 22, washed in Tyrode solution, and fresh medium added. Jan. 25, same treatment. Jan. 28, fragments of coagulum transferred to cover-glasses and stained; camera lucida drawings. Length of cells, 30μ; active kinoplasm; trophoplasm filled with large number of neutral red vesicles, deeply stained, resembling berries; diameter of vesicles, 1 to 4μ. Vesicles contained many small granules, irregular in shape, and darkly stained; no rosette arrangement.

Experiment 5. Blood Monocytes Cultivated for 13 Days.—Culture 2954-C, Jan. 7. Fragments of leucocytic film from normal chickens cultivated on cover-glasses in equal parts of plasma and embryo juice; three passages into fresh medium. Jan. 19, stained; camera lucida drawings. Very long and slender monocytes; active kinoplasm; length of cells, 60μ; 15 neutral red vesicles, 0.5 to 2μ in diameter. Some of the vesicles contain 1 to 5 small red granules.

Experiment 6. Tissue Macrophages after 24 Hours Cultivation.—Culture 3136-C5, Feb. 1. A fragment of subcutaneous connective tissue from a normal adult chicken cultivated on cover-glasses in equal parts of plasma and embryo juice; migration of a few cells. Feb. 2, stained; camera lucida drawings. Length of cells, 30μ; active kinoplasm. Trophoplasm contains 8 or 10 neutral red vesicles, 1 to 2μ in diameter, each vesicle having several large red granules, generally agglutinated together; some fat globules between the vesicles.

Experiment 7. Tissue Macrophages after 24 Hours Cultivation.—Culture 2956-C5, Jan. 14. Fragments of subcutaneous connective tissue from an adult chicken cultivated on cover-glasses in equal parts of plasma and embryo juice. Jan. 15, stained; camera lucida drawings. Long, slender macrophages; very active flagellate pseudopods; length of cells, 60 to 85μ; about 8 neutral red vesicles, 0.5 to 2μ in diameter, containing 1 or 2 small neutral red granules.

Experiment 8. Tissue Macrophages after 24 Hours Cultivation.—Culture 3097-C, Jan. 28. Fragments of subcutaneous connective tissue from an adult chicken cultivated on cover-glasses in equal parts of plasma and embryo juice. Jan. 29, stained; camera lucida drawings. Diameter of cells, 30μ; approximately 30 moderately stained neutral red vesicles, 0.5 to 3μ in diameter. Most of the vesicles contain coarse, dark red granules which move more or less actively; many small fat globules between the vesicles. After a few minutes, the fluid in the vacuoles takes up the stain gradually and becomes orange, yellow, or red; vesicles not arranged around the centriole, but distributed through the trophoplasm; active folding and unfolding of the kinoplasm.

Experiment 9. Tissue Macrophages after 24 Hours Cultivation.—Culture 3777-C, Apr. 22. A fragment of subcutaneous connective tissue from a normal adult chicken cultivated on cover-glasses in equal parts of plasma and embryo juice. Apr. 23, migration of a large number of macrophages; stained; camera lucida drawings. Diameter of cells, 40μ; active kinoplasm. Trophoplasm contains about 20 neutral red vesicles, 0.25 to 2μ in diameter, showing rosette disposition; small fat globules around nucleus and vesicles; mitochondria deeply stained, globular, and rod-shaped (Fig. 9).

Experiment 10. Tissue Macrophages after 24 Hours Cultivation.—Culture 3790-C, Apr. 23. A fragment of subcutaneous connective tissue from a normal adult chicken cultivated in equal parts of plasma and embryo juice. Apr. 24, migration of moderate number of macrophages; stained; camera lucida drawings. Length of cells, 75μ; active kinoplasm; trophoplasm filled with neutral red vesicles, 0.25 to 2μ in diameter; no fat globules; mitochondria deeply stained, globular, and rod-shaped (Fig. 10).

In a second series of experiments, blood monocytes and fragments of subcutaneous connective tissue from adult chickens were cultivated in a control medium composed chiefly of serum, and an experimental medium of Tyrode or Ringer solution. The cover-glass preparations were stained with neutral red or neutral red and Janus green.

Experiment 1. Effect of Tyrode Solution and Serum on Monocytes.—Culture 3386-C, Mar. 2. Fragments of leucocytic film cultivated in flasks in the ordinary medium composed of 0.5 cc. plasma, 0.5 cc. embryo juice, and 1 cc. Tyrode solution. Mar. 3, cultures washed with 2 cc. of Tyrode solution, and patched with 25 cc. plasma and 25 cc. embryo juice. Mar. 4, cultures washed in 2 cc. Tyrode

solution, which is removed after 10 minutes; Flask 1, 1 cc. Tyrode solution introduced, and Flask 2, 0.5 cc. serum and 0.5 cc. Tyrode solution. Mar. 6, cultures washed in Tyrode solution and fresh fluid introduced into the flasks. Mar. 8, fragments of coagulum transferred to cover-glasses; stained; camera lucida drawings.

1. Monocytes in Tyrode solution. Length of cells, 32μ; active kinoplasm; trophoplasm filled with faintly stained neutral red vesicles containing no granules; between the vesicles, some fat globules and faintly stained mitochondria. The latter are small dots, hardly visible (Fig. 11).

2. Monocytes in serum. Very active multibranched cells, 50μ in length; kinoplasm packed with smaller neutral red vesicles, deeply stained, resembling berries; practically no fat globules; well stained mitochondria, globular, rod-shaped, and filamentous (Fig. 12).

Experiment 2. Effect of Tyrode Solution on Monocytes.—Culture 3079-A1, Feb. 18. Monocytes which have grown in the ordinary medium since Feb. 16 are washed for 10 minutes with 2 cc. Tyrode solution. After its removal, 1 cc. of Tyrode solution is introduced into the flask. Feb. 21, same treatment. Feb. 24, fragment of coagulum transferred to cover-glasses; stained; camera lucida drawings. Macrophages are of small size with very active kinoplasm; length of cells, 25μ. The area occupied by the neutral red vesicles and the nucleus is very small. About 15 small neutral red vesicles, 0.25 to 1μ, are grouped around the centriole. Between them and the centriole is an area lightly stained in red and composed of small granules; rosette arrangement; faintly stained mitochondria as small dots around the nucleus and the neutral red vesicle; no fat.

Experiment 3. Effect of Ringer Solution and Serum on Monocytes.—Culture 3445-C, Mar. 9. Preparation of the cultures in the ordinary medium. Mar. 11, cultures washed in 2 cc. Ringer solution. After the removal of the fluid, 1 cc. Ringer solution is introduced into Flask 1, and 0.5 cc. serum and 0.5 cc. Ringer solution into Flask 2. Mar. 12 and 13, same treatment. Mar. 15, fragments of coagulum transferred to cover-glasses; stained; camera lucida drawings.

1. Monocytes in Ringer solution. Small cells, 25μ in length; 25 pale vesicles, 0.25 to 1μ in diameter, and faintly stained mitochondria; large number of small fat globules; active kinoplasm (Fig. 13).

2. Monocytes in serum. Cells active and larger, 50μ in length; area occupied by the neutral red vesicles, 0.25 to 3μ in diameter, containing a fluid more deeply stained than in cells cultivated in Ringer solution; few fat globules; large number of mitochondria (Fig. 14).

Experiment 4. Effect of Ringer Solution and Serum on Monocytes.—Culture 3471-C, Mar. 11. Preparation of the cultures by the ordinary method. Mar. 13, cultures washed in 2 cc. Tyrode solution. After removal of the fluid, 1 cc. of Ringer solution is introduced into Flask 1, and 0.5 cc. serum and 0.5 cc. Ringer solution into Flask 2. Mar. 15, cultures washed and fresh medium introduced. Mar. 16, fragments of coagulum transferred to cover-glasses; stained; camera lucida drawings.

1. Monocytes in Ringer solution. Elongated and very active cells, 25μ in length; about 15 faintly stained neutral red vesicles, 0.25 to 1.25μ in diameter, scattered through the trophoplasm, and with no granules; mitochondria as small dots, faintly stained (Fig. 15).

2. Monocytes in serum. Very active cells, with several branches, 40μ in length; deeply stained neutral red vesicles, 0.25 to 1.25μ in diameter, without granules, arranged around centriole; globular and rod-like mitochondria, well stained (Fig. 16).

Experiment 5. Effect of Tyrode Solution and of Blood Serum on Monocytes.—Culture 8496-D, Feb. 1. Fragments of culture of monocytes 3030-A2 are placed in flasks, containing 1.5 cc. plasma and 0.5 cc. embryo juice. After coagulation, Flask 1 washed for 10 minutes in 4.5 cc. of Tyrode solution. After the fluid is removed, 1.5 cc. of Tyrode solution is introduced into the flask. Flask 2 is left undisturbed. Feb. 2, Flask 1 washed with Tyrode solution for 10 minutes. Feb. 3, Flask 1 washed for 5 minutes with Tyrode solution; surface of coagulum of Flask 2 washed for a few seconds in Tyrode solution, and 0.5 cc. serum introduced. In Flask 1, monocytes are elongated, transparent, and in comparatively small numbers. In Flask 2, they are shorter, less transparent, very numerous, and invading the medium actively. Feb. 5, fragments from Flask 2 transferred to cover-glasses; stained; camera lucida drawings. Feb. 6, fragments from Flask 1 transferred to cover-glasses; stained; camera lucida drawings.

1. Monocytes in Tyrode solution. Very active cells, 35μ in length; small segregation apparatus; a few neutral red vesicles, 0.25 to 0.75μ in diameter, faintly stained; no granules; few fat globules.

2. Monocytes in serum. Very active cells, 50μ in length; very large segregation apparatus; many neutral red vesicles, 0.5 to 2μ in diameter, resembling a mass of darkly stained berries; no fat globules.

Experiment 6. Effect of Tyrode Solution and Blood Serum on Monocytes.—Culture 3511-C, Mar. 16. Fragments of leucocytic film cultivated in the ordinary manner. Mar. 18, cultures washed with 2 cc. Tyrode solution for 10 minutes, 1 cc. Tyrode solution introduced into Flask 1, and 1 cc. of 50 per cent serum into Flask 2. Mar. 19, cultures washed with 2 cc. Tyrode solution for 10 minutes, and fresh fluid introduced into each flask. Mar. 20, fragments transferred to cover-glasses; stained; camera lucida drawings.

1. Monocytes in Tyrode solution. Cells, 25μ in diameter; active kinoplasm; faintly stained neutral red vesicles, 0.25 to 0.5μ in diameter; few mitochondria, faintly stained; a few small fat globules.

2. Monocytes in serum. Length of cells, 55μ; neutral red vesicles, 0.25 to 3μ in diameter, more deeply stained; mitochondria well stained; a number of small fat globules scattered about among the vesicles.

Experiment 7. Effect of Tyrode Solution and Serum on Monocytes.—Culture 3020-A, Jan. 21. Fragments of leucocytic film cultivated in the ordinary manner. After coagulation, 1 cc. Tyrode solution introduced into Flask 1, and 0.5 cc. serum and 0.5 cc. embryo juice into Flask 2. Jan. 22, cultures washed with 2 cc. Tyrode

solution for 10 minutes, and after removal of the fluid, patched with 1 cc. of 0.5 cc. plasma and 0.5 cc. embryo juice. After coagulation, fresh fluid added to each flask. Jan. 23, same treatment. Jan. 27, fragments from Flask 1 transferred to cover-glasses; stained; camera lucida drawings; Flask 2, ordinary treatment. Jan. 28, fragments from Flask 2 transferred to cover-glasses; stained; camera lucida drawings.

1. Monocytes in Tyrode solution. Length of cells, 30μ; small vesicular apparatus; about 20 neutral red vesicles, 0.25 to 1.5μ in diameter, faintly stained; no granules in the vesicles; about 15 fat globules.

2. Monocytes in serum and embryo juice. Length of cells, 40μ; about 40 neutral red vesicles, 0.25 to 2.5μ in diameter, many of them containing red granules.

Experiment 8. Effect of Tyrode Solution on Tissue Macrophages.—Culture 3841-C, Apr. 29. Fragments of subcutaneous connective tissue from an adult chicken cultivated on cover-glasses in equal parts of plasma and embryo juice. Apr. 30, extensive migration of macrophages into medium; one preparation stained; camera lucida drawings. Diameter of cells, 45μ; many neutral red vesicles, 0.25 to 3.5μ in diameter; rosette disposition; no fat globules; numerous mitochondria, well stained granules and short rods grouped around nucleus and vesicular area (Fig. 17).

Other preparations transferred to a flask containing 0.5 cc. plasma, 1 cc. Tyrode solution, and 0.5 cc. embryo juice. After coagulation, culture washed in 2 cc. Tyrode solution for 10 minutes; fluid removed, and 1 cc. Tyrode solution introduced into the flask. May 1, a few active cells migrating into the fresh medium; washed in 2 cc. Tyrode solution, and fresh Tyrode solution introduced. May 3, same treatment. May 5, fragments of coagulum transferred to cover-glasses; stained; camera lucida drawings. Very active cells, 25μ in diameter; neutral red vesicles, 0.25 to 1.5μ in diameter; rosette disposition; no fat globules; mitochondria well stained granules with a few rods grouped around nucleus and vesicular area (Fig. 18).

The results of these two series of experiments may be summarized as follows:

In the first series, blood monocytes and tissue macrophages were cultivated in identical media for various periods of time. The monocytes, examined 2 hours after the preparation of the cultures (Figs. 3 and 4), were 10 to 15 μ in length. Their undulating membrane moved unceasingly and showed its folds as flagellate pseudopods. The neutral red vesicles, 6 to 10 in number, were radially arranged around the centriole, or scattered irregularly through the trophoplasm. The mitochondria were small and round (Figs. 3 and 4). After 24 hours (Fig. 5), the size of the cells increased, their length

reaching 30 to 40 μ. The neutral red vesicles were augmented in number and size, and filled with orange-red fluid containing small or large dark granules. No fat globules were seen. The mitochondria assumed the appearance of rods or short filaments. Every monocyte present in the preparation was transformed in this manner. The change became more marked after 48 hours (Fig. 6). The trophoplasm was filled with debris of polymorphonuclear leucocytes. After 72 hours (Fig. 7), the size of the cells reached 75 μ. The segregation apparatus and the mitochondria showed a corresponding increase. The undulating membrane was very active. The polymorphonuclear leucocytes were normal (Fig. 1). However, a number of them and also some red blood corpuscles had been phagocyted and were being digested by the monocytes. All the monocytes present in the preparation had become large cells, indistinguishable from tissue macrophages. After 96 hours cultivation (Fig. 8) the polymorphonuclear leucocytes, lymphocytes, and red corpuscles had practically disappeared from the preparation, and the segregation apparatus of the monocytes had slightly decreased in size. The undulating membrane moved only at the ends of the cells.

The tissue macrophages were always larger than the monocytes just obtained from the blood. Their size varied from 30 to 85 μ. The undulating membrane and its flagellate-like folds moved in the same manner as those of the monocytes. The number of the neutral red vesicles varied from 15 to 30 or more. They were often grouped around the centriole in a typical rosette. Their diameter varied from 1 to 2 μ. Generally, they contained 1 or 2 small red granules. A few fat globules were seen between the vesicles. The mitochondria were located around the nucleus and the mass of the vesicles, and assumed the shape of dots and rods (Figs. 9 and 10).

As these results clearly show, blood monocytes increase in size when cultivated in plasma and embryo juice in the presence of polymorphonuclear leucocytes, and in 24 hours they reach the dimensions of the tissue macrophages. At the same time that the size and proliferating activity of the cells are augmented, the segregation apparatus becomes larger, dark stained granules appear in the vesicles, and the mitochondria assume the shape of rods and short filaments. The rosette arrangement of the neutral red vesicles around the centriole

is present in the tissue macrophages, as well as in the monocytes. However, it is not a constant characteristic of either of these cells. There is no difference between the mitochondria of monocytes and of macrophages, after the monocytes have been cultivated in plasma and embryo juice. As all the large mononuclear cells present in the medium exhibit a similar appearance and size, they certainly derive from the monocytes observed in the fresh cultures. It is obvious that, when cultivated under the same conditions, the blood monocytes become indistinguishable from the macrophages of adult connective tissue (Figs. 7 to 10).

In the second series of experiments, macrophages originating from monocytes and tissue macrophages were placed in nutrient and non-nutrient media, in order to ascertain whether their morphology could be modified by a nutritional change. It is known that serum is a nutrient medium for the monocytes, whereas they starve in Tyrode or Ringer solution. The control monocytes, which were kept in serum, continued to resemble tissue macrophages (Figs. 12, 14, and 16). The segregation apparatus was well developed and the vesicles often looked like red berries and contained some granules. The mitochondria appeared as short filaments, deeply stained with Janus green. The cells remained large and very active. Their length varied from 40 to 70 μ. The experimental monocytes were deprived of food in flasks containing only Tyrode or Ringer solution as a fluid medium (Figs. 11, 13, and 15). Their size decreased, and the nucleus also became smaller. At the same time, the segregation apparatus lost its importance, the granules disappeared from the vesicles which were filled with pale fluid, and the mitochondria became shorter, less numerous, and faintly stained. Some fat globules appeared between the vesicles. It was evident that in the cells subjected to starvation, the neutral red vesicles and the mitochondria became profoundly modified. Similar phenomena were observed in tissue macrophages cultivated in a medium composed chiefly of Tyrode solution (Figs. 17 and 18). They reverted to a smaller type, markedly resembling monocytes.

Monocytes and tissue macrophages do not differ essentially from one another. They represent functional states of a single type, which readily responds to a change in nutrition by modifying its protoplasmic

organs. Cell morphology is determined by, and varies according to, the chemical composition of the pericellular fluid.

Mode of Locomotion of the Macrophage and Structure of Its Kinoplasm.

The mode of locomotion of the macrophage was studied on cinematographic films of cultures of blood, connective tissue, and Rous sarcoma. Both direct light and dark-field illumination were used. The photographs were taken at a speed of 60 exposures per minute and at a magnification of 400 diameters.[3] As the films were projected at a speed of 10 or 11 exposures per second, the rate of motion of the cells was increased approximately ten times. It was thus possible to observe simultaneously on the films the progression of the cells and the movements of their organs. The small monocytes observed in freshly prepared cultures moved more slowly than the polymorphonuclear leucocytes, and more rapidly than the lymphocytes. The mode of progression of the three types of cells differed widely. The lymphocytes sent forth short, blunt pseudopods, and advanced like small worms. The polymorphonuclear leucocytes projected forward long arms, into which the rods streamed. Often they dragged a thin tail behind them. While the polymorphonuclear leucocytes moved like amebæ, the monocytes assumed an appearance more nearly comparable to that of an octopus. Around their body and processes, thread-like pseudopods moved unceasingly. They could be distinguished at first sight from the polymorphonuclear leucocytes and lymphocytes by their peculiar way of carrying themselves. After the monocytes had assumed the appearance of macrophages, their mode of locomotion was not modified. The movements of both monocytes and macrophages growing from adult connective tissue were identical, and neither cell could be distinguished from the other.

The study of macrophages photographed by direct light and by dark-field illumination led to a clear conception of their structure. These cells are composed, as is well known, of a central body or trophoplasm, and a marginal portion or kinoplasm. The trophoplasm contains the nucleus, the mitochondria, and the neutral red vesicles. Although the cell body is plastic, and the nucleus as well as the cyto-

[3] The cultures were filmed by Mr. Heinz Rosenberger.

plasmic organs glide nimbly into the advancing process during the progression of the cell, it is relatively immobile when compared to the kinoplasm. The kinoplasm is the peripheral and thin cytoplasm from which thread-like pseudopods seem to extend themselves. These filiform appendages move with great activity. When the flagellated pseudopods are examined on films taken with dark-field illumination, they appear to be the folds of an extremely thin membrane which projects itself from the kinoplasm and is a part of it. This membrane undulates incessantly, like a delicate silk veil when blown by the wind. Under direct light, the membrane becomes invisible. Its folding and outer edges only are then seen, to be mistaken for thread-like pseudopods. When the macrophage stops progressing and assumes a circular shape, it appears surrounded by the membrane which completely unfolds itself and moves like the waves of the sea on a sandy shore. Under dark-field illumination, the presence of the membrane is detected because its surface as well as its folds become lighted. When, in the course of a slow undulation, the convex surface of a fold is illuminated from the side, the membrane is seen as an extremely thin structure covering part or all of the periphery of the cell, and molding itself upon any impinging bodies, fibrin, bacteria, and other cells. When a lymphocyte approaches a macrophage, it is rapidly enveloped by the foldings and appears to glide into them toward the body of the cell, where it is digested. The incessant motion of the undulating membrane is probably the cause of the inability of the macrophages to unite and form a tissue as fibroblasts do. Possibly it is an essential function of the cytoplasm, and its suppression, when cells are crowded, may bring about death.

Macrophages obtained from the subcutaneous connective tissue of adult chickens were studied by the same method, and found to move exactly as do monocytes. Normal blood monocytes, large monocytes growing in flasks, sarcoma cells, and tissue macrophages possess an undulating membrane. It is a characteristic common to the four groups of cells.

Characteristics of the Colonies of Macrophages.

When fragments of a leucocytic film are placed in a medium composed of 0.5 cc. plasma, 1 cc. Tyrode solution, and 0.5 cc. embryonic

extract, ameboid cells migrate in large numbers into the coagulum. The polymorphonuclear leucocytes, which move rapidly, occupy the outer zone of the invaded area, while the inner zone consists chiefly of the slower monocytes which later multiply and scatter everywhere. Eventually, small islands composed of monocytes appear at a distance from the main colonies. The monocytes rapidly phagocyte the polymorphonuclear leucocytes and in 3 or 4 weeks spread on the entire medium in a more or less regular formation. They do not build up a tissue, as fibroblasts do. Sometimes they unite as chains. They never come into contact at their sides. Instead of remaining packed together, they disperse through the whole coagulum. They may congregate around some foreign bodies, such as fragments of muscle or protein precipitate, which they devour. But as soon as the food is exhausted, they scatter again. When the original fragments of the leucocytic film have been removed, the coagulum appears as homogeneous. As long as the cells remain normal, the flask seems to be empty. When the segregation apparatus develops abnormally, the colonies may look like whitish spots on the transparent background. If death occurs, the colonies become opaque. Digestion of the coagulum does not take place even after the death of the cells. As a rule, digestion of the coagulum down to the glass is a characteristic of the malignant transformation of the cells after inoculation with Rous virus. The clot seems to be moth-eaten, and on the edges of the digested areas small masses of necrotic tissue may be seen. Simultaneously, some cells assume the appearance of fibroblasts. The transformation of the macrophages into fibroblasts never occurs spontaneously when the cells are allowed to migrate freely, as they do in a normal culture and when no sarcoma virus is present. If macrophages crowd together through the action of some mechanical factor, or following inoculation with Rous virus, they die or become transformed into fibroblasts.

Colonies of tissue macrophages were obtained from the subcutaneous connective tissue of adult chickens, and from other normal or pathological tissues. Their appearance and behavior have always proved to be identical with those of the monocytes.

Rate of Growth of the Colonies.

In a medium composed chiefly of blood serum, the rate of multiplication and migration is such that monocytes originating from fragments of a film about 3 by 2 mm. entirely cover the coagulum of a flask 5 cm. in diameter in approximately 3 weeks. The rate of growth of the colony may be appraised in terms of the time the cells take to invade the culture medium and the number of cells contained in the unit of surface. However, no method has yet been developed by which the rate of multiplication may be accurately measured. Only considerable differences can be appreciated. There is no difficulty in detecting the variations in the rate of multiplication of macrophages or monocytes in media widely different in nature. When colonies of monocytes are cultivated in a hanging drop and transferred to a new medium every 2 or 3 days, the size of the colonies generally doubles in 4 or 5 days. The velocity of the multiplication of the cells is probably less great than that of epithelial cells and fibroblasts.

Food Requirements of Macrophages. Their Susceptibility to Arsenous Oxide.

Monocytes and macrophages do not feed on egg albumin, nucleic acid, amino acids, crystallized egg albumin, egg yolk, or pure embryo juice. They have the property of living and multiplying in blood serum and differ from fibroblasts and epithelial cells, which die in blood serum and multiply indefinitely in embryo juice. Although they may proliferate for several weeks when cultivated in plasma or serum, it is far from certain that they reach their optimum metabolic activity in this medium. In fresh blood cultures, monocytes almost immediately phagocyte polymorphonuclear leucocytes and red corpuscles, and their length increases from 10 or 15 μ to 70 or 80 μ. When the food is exhausted, they generally revert to a smaller type, even in the presence of blood serum. The increase in size of the cells during the first days of cultivation is probably due to the abundant food supplied by polymorphonuclear leucocytes, lymphocytes, and red corpuscles. Monocytes and macrophages also absorb with avidity minute fragments of dead muscle, or particles of precipitated

protein. Fragments of muscle, killed by heating or freezing and placed in the immediate vicinity of a colony of monocytes, are soon invaded by migrating cells which creep along them and begin their work of erosion. The muscle fibers assume the appearance of worm-eaten logs. They finally disintegrate under the repeated attacks of the monocytes, which migrate through the medium heavily charged with debris. As long as the cells congregate around the muscle fibers, they remain very large. After the supply of food is exhausted, they become smaller. A similar phenomenon takes place when drops of a protein precipitate are deposited near a group of monocytes. It seems as if discrete particles of proteins are better food than proteins in solution. However, when the fluid medium contains a minute amount of proteose, the rate of proliferation is also markedly augmented. If a larger concentration of proteose is used, the monocytes accumulate many fat globules and neutral red vacuoles, and die after a short time. It is significant that a concentration of proteose, which greatly increases the rate of growth of the fibroblasts, brings about the death of the macrophages.

Monocytes are endowed with a peculiar resistance to the action of arsenous oxide. When they are cultivated for 24 hours in a medium containing arsenous oxide at a concentration of 1/180,000, they die in large numbers. However, some of the cells resist the poison and multiply actively. A concentration of 1/200,000 is much less injurious. The cells proliferate in spite of having been treated by arsenous oxide, and after a few days the cultures assume a normal appearance. While macrophages resist arsenous oxide at a concentration of 1/200,000, fibroblasts are killed when their medium contains arsenous oxide at a concentration of 1/800,000. If fragments of Rous sarcoma are placed in a medium containing 1/400,000 arsenous oxide, the fibroblasts and the degenerating sarcoma cells rapidly disappear and only normal macrophages survive. Tissue macrophages possess approximately the same food requirements and the same susceptibility to arsenous oxide as blood monocytes.

DISCUSSION.

The essential properties of the blood monocyte and the tissue macrophage appear to be identical, as shown by the appearance of the

colonies, their action on the medium, the mode of locomotion and the structure of the cells, their rate of growth, their food requirements, and their susceptibility to certain toxic substances. The cells differ slightly in the size and development of their segregation apparatus and mitochondria (Figs. 3 to 10). The radial arrangement of the neutral red vacuoles around the centriole, or rosette disposition, considered by Sabin, Doan, and Cunninghan[4] as being characteristic of the monocyte (Figs. 3 and 16), is frequently observed also in the tissue macrophage (Figs. 9, 17, and 18). In normal macrophages, as well as in monocytes, mitochondria are always present (Figs. 12 and 17). Monocytes and macrophages cultivated in media of identical composition become identical in structure. There is no difference between a monocyte that has been kept for a few days in plasma and embryo juice, and a tissue macrophage (Figs. 5, 7, and 10). Both cells have approximately the same size; their segregation apparatus is well developed; the neutral red vesicles may be arranged in a rosette around the centriole or scattered through the cytoplasm; and the mitochondria appear as short filaments. The disposition of the neutral red vesicles around the centriole, their size and number, the condition of the mitochondria, and the presence of the fat globules, are determined by the composition of the culture medium and not by the origin of the cells. A monocyte supplied with an abundance of food in a solid medium becomes a macrophage (Fig. 7). A macrophage is merely a monocyte in a more active metabolic condition.

The polymorphism of the cell was further ascertained by experiments in which macrophages derived from monocytes or obtained from adult connective tissue were caused to revert to a less active metabolic state. When blood macrophages (Figs. 12, 14, and 16) were cultivated in Tyrode or Ringer solution, the starved cells became smaller (Figs. 11, 13, and 15), the rod-like mitochondria assumed the appearance of dots, the neutral red vesicles decreased in number and diameter, and their segregation granules disappeared. A similar phenomenon was observed when tissue macrophages (Fig. 17) from the subcutaneous connective tissue of adult chickens were cultivated in nutrient and

[4] Sabin, F. R., Doan, C. A., and Cunningham, R. S., *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 125.

non-nutrient media. The starved tissue macrophages grew smaller (Fig. 18), lost part of their segregation apparatus, reduced their mitochondria, and became almost similar to monocytes freshly removed from the blood. It is obvious that macrophages can revert to a form closely resembling monocytes, when they are deprived of food. These facts completed the demonstration that monocytes and macrophages are the expression of various metabolic states of a single type. Monocytes, macrophages, and fibroblasts are closely related forms. While the transformation of a monocyte into a macrophage is easily brought about by an abundant food supply, that of a macrophage into a fibroblast requires the presence of substances of unknown nature, probably set free by dead cells.[5]

Between monocytes and macrophages, the main difference is one of size (Figs. 3 and 10). In both cells, the mode of locomotion and the movement of the cytoplasmic organs are identical. Both cells are surrounded by an undulating membrane which moves unceasingly (Figs. 6 and 9). The external boundary of this appendage is irregular and invisible with direct light. This characteristic distinguishes the macrophage from the fibroblast which is limited by a sharp line of geometric design. The surface of the fibroblast is rigid, and the motion of the protoplasm takes place only at the end of the processes. When a macrophage begins to lose its activity, its sides become limited by a sharp, regular line, while the undulating membrane remains present at both ends (Fig. 8). The structural characteristics of fibroblasts and macrophages which are clearly noted in cinematographic films explain the differences observed in the constitution of the colonies. The fibroblasts that move only through their end processes can come into close contact at their sides. They form circular colonies where each component cell flows radially toward the periphery. On the contrary, macrophages are incapable of remaining packed together because they are surrounded by a membrane which moves continuously. They progress far more rapidly than the fibroblasts, and scatter through the coagulum without forming any dense colonies.

Both macrophages and fibroblasts possess a segregation apparatus which increases in size when the cells receive an abundant food supply

[5] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, xliii, 461.

(Figs. 3, 6, and 7). At the same time, the mitochondria augment in length and number. The segregation apparatus of the macrophage is far larger than that of the fibroblast on account of the greater metabolic activity of the former cell. When macrophages are fed on certain split products of proteins, or on fragments of dead muscle, they multiply more actively and develop more numerous and larger neutral red vesicles which generally contain small granules. If they are starved in Tyrode or Ringer solution, the neutral red vesicles lose their granules, decrease in size and number, and the cells themselves become smaller. In the macrophage, as in the fibroblast, the development of the segregation apparatus and of the mitochondria depends on the composition of the pericellular fluid.

The ability of the macrophage to multiply when fed upon blood serum[6] or particles of proteins, and the fact that it dies in pure embryo juice, sharply distinguish this cell from the fibroblast. The fibroblast stops multiplying in blood serum and does not phagocyte other cells and fragments of proteins. It may feed upon substances set free in the medium by leucocytes[6] and thyroid gland, and proliferate indefinitely in plasma and embryonic juice. A similar phenomenon occurs with proteose which, at a certain concentration, promotes the growth of fibroblasts and kills the macrophages.[7] A third property distinguishing the macrophage from the fibroblast is a different susceptibility of the cells to arsenous oxide. The fibroblast dies in a concentration of arsenous oxide which is completely innocuous for the macrophage.[8] The causes and the mechanism of the contrasting characteristics of the two types of connective tissue cells are still unknown.

It is certain that the innate physiological properties of the macrophages determine their behavior within the organism. The knowledge of these fundamental characteristics necessarily throws some light on the mechanism of the phenomena in which the cells participate. Macrophages are rarely present in the blood,[9] and monocytes are not

[6] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 513.

[7] Carrel, A., *Compt. rend. Soc. biol.*, 1926, xciv, 1060. Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xliv, 387, 397.

[8] Carrel, A., unpublished experiments.

[9] Rowley, M. W., *J. Exp. Med.*, 1908, x, 78. Simpson, M. J., *J. Med. Research*, 1922, xliii, 77.

found in the subcutaneous connective tissue. In the blood, as well as in the tissues, the structure of the mononuclear cell depends upon the composition of its medium. It is probable that the nutrition of spherical monocytes in suspension in a fluid is not as active as when the cells, stretching themselves on the fibrin framework of a coagulum, greatly increase their surface. The only response that the large mononuclear can make to the physicochemical conditions of the circulating blood is probably to assume a monocytic form. On the other hand, when monocytes migrate from the capillaries into the tissues, they automatically become transformed into macrophages, as they do in a solid medium containing an abundant food supply.

The abnormal multiplication of monocytes and macrophages in certain diseases is probably due to an increase in their food supply. The formation of an exudate richer in proteins than normal lymph, the death of leucocytes, lymphocytes, or red corpuscles, the presence of blood cells in the tissues, the destruction of groups of cells by toxins, bacteria, and parasites, certain split products of proteins, etc., may give to monocytes and macrophages the food required for their proliferation and bring about monocytosis, as well as local accumulation of macrophages in the tissues. Like amebæ, macrophages utilize for their nutrition the nitrogenous particles that they phagocyte. The various substances taken up by the cells are elaborated in their rhagiocrin apparatus.[10] The neutral red vesicles increase in size and segregate a number of granules, which disappear if the colonies are starved. The substances manufactured by the rhagiocrin organs are probably used by the cell itself, or set free in the medium. Some of the products of the macrophages consist of substances which promote the multiplication of epithelial cells and fibroblasts[11] and are analogous to those present in embryo juice. Monocytes may secrete these substances in the culture medium[12] and stimulate the growth of colonies of fibroblasts.[6] They transform proteins from serum and from the nitrogenous material of living or dead cells into food which they may bring to fixed tissue cells.[11] The nature of their secretions depends

[10] Renaut, J., *Arch. anat. micr.*, 1906–07, ix, 495.
[11] Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385.
[12] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 645.

possibly upon the composition of the substances they ingest. It is probable that the products of macrophages fed upon muscle fibers or bacteria are not identical. Antibodies may be a mere by-product of the nutrition of the macrophage. However, it is not known in what measure the cells use bacterial proteins for their own nutrition or even whether they use them at all. They can simultaneously secrete natural hemolysin and growth-promoting substances for epithelial cells and fibroblasts,[13] oppose a foreign invader,[14] and stimulate the tissues of the organism. The secretion of growth-promoting substances may explain the resumption of the activity of epithelial cells and fibroblasts in cicatrization, regeneration, sclerosis of organs, and tumor growth.[11] However, proliferation of resting fibroblasts and epithelial cells can also be determined by certain split products of the protein molecule.[7] The proliferation of cells within the inhibiting humors of the adult organism may be due to the production of proteoses by degenerating material as well as to the setting free in the tissues of proteins of the embryonic type. The secretion of ferments is not observed to take place in pure cultures of macrophages. The macrocytase of Metchnikoff remains inside of the cells or, if it is set free, the conditions of the medium do not allow any manifestation of its activity. When macrophages are transformed into malignant cells by Rous virus,[15] they digest the fibrin of the medium and give to the coagulum its characteristic appearance. Proteolysis only occurs around sick or dead macrophages.

There is no doubt that the function of the macrophages is far more important than was thought by Metchnikoff. In some cases, they are the defenders of the organism against infections, but they may also be the direct source of the invasion of the tissues by a disease such as Rous sarcoma.[16] The destruction by phagocytosis of cells and bacteria, the secretion of antibodies, the reproduction of Rous virus, and the genesis of sarcoma cells, are phenomena of secondary importance,—a pathological adaptation of general physiological properties. The main function of the macrophages is related to the nutrition of the

[13] Carrel, A., *J. Am. Med. Assn.*, 1924, lxxxii, 255.
[14] Gay, F. P., and Morrison, L. F., *J. Infect. Dis.*, 1923, xxxiii, 338.
[15] Carrel, A., *J. Am. Med. Assn.*, 1925, lxxxiv, 157.
[16] Carrel, A., *Paris. méd.*, 1926, lix/lx, 274.

tissues. Their dual and opposite rôles in the war on, and the propagation of, disease are accessory phenomena.

This study of the biological characteristics of the fibroblast and the macrophage is still very incomplete. However, the methods by which this important aspect of cytology may profitably be investigated have been built up and can be widely used. They will give rise to a thorough knowledge of the fundamental properties of both types of connective tissue cells, and ultimately reveal the mechanism of the normal and pathological phenomena in which these cells are involved.

CONCLUSIONS.

1. Monocytes and tissue macrophages become identical in appearance when they live under identical conditions. Macrophages cultivated in nutrient and non-nutrient media acquire different structures. Monocytes and tissue macrophages are mere functional variations of a single type.

2. The structure of the segregation apparatus and of the mitochondria of monocytes and macrophages depends on the composition of the pericellular fluid and on the metabolic state of the cells.

3. The monocyte and the macrophage are endowed with a number of physiological properties which become apparent when they grow in pure cultures.

4. The knowledge of these fundamental characteristics explains the behavior of the cells within the organism.

EXPLANATION OF PLATES.

The yellow tone in the original drawings is omitted in reproduction.

PLATE 8.

FIG. 1. Culture 8740-D7. Polymorphonuclear leucocyte after 2 hours cultivation in plasma and embryo juice.

FIG. 2. Culture 8740-D7. Red blood corpuscle.

FIG. 3. Culture 8740-D7. Blood monocyte after 2 hours cultivation in plasma and embryo juice.

FIG. 4. Culture 8741-D1. Blood monocyte after 2 hours cultivation in plasma and embryo juice.

FIG. 5. Culture 8740-D6. Blood monocyte after 24 hours cultivation in plasma and embryo juice.

FIG. 6. Culture 8740-D14. Blood monocyte after 48 hours cultivation in plasma and embryo juice.

FIG. 7. Culture 8740-D15. Blood monocyte after 72 hours cultivation in plasma and embryo juice.

FIG. 8. Culture 8740-D19. Blood monocyte after 96 hours cultivation in plasma and embryo juice.

FIG. 9. Culture 3777-C. Tissue macrophage after 24 hours cultivation in plasma and embryo juice.

FIG. 10. Culture 3790-C. Tissue macrophage after 24 hours cultivation in plasma and embryo juice.

PLATE 9.

FIG. 11. Culture 3386-C1. Blood monocyte cultivated in Tyrode solution for 4 days.

FIG. 12. Culture 3386-C2. Blood monocyte cultivated in serum for 4 days.

FIG. 13. Culture 3445-C1. Blood monocyte cultivated in Ringer solution for 4 days.

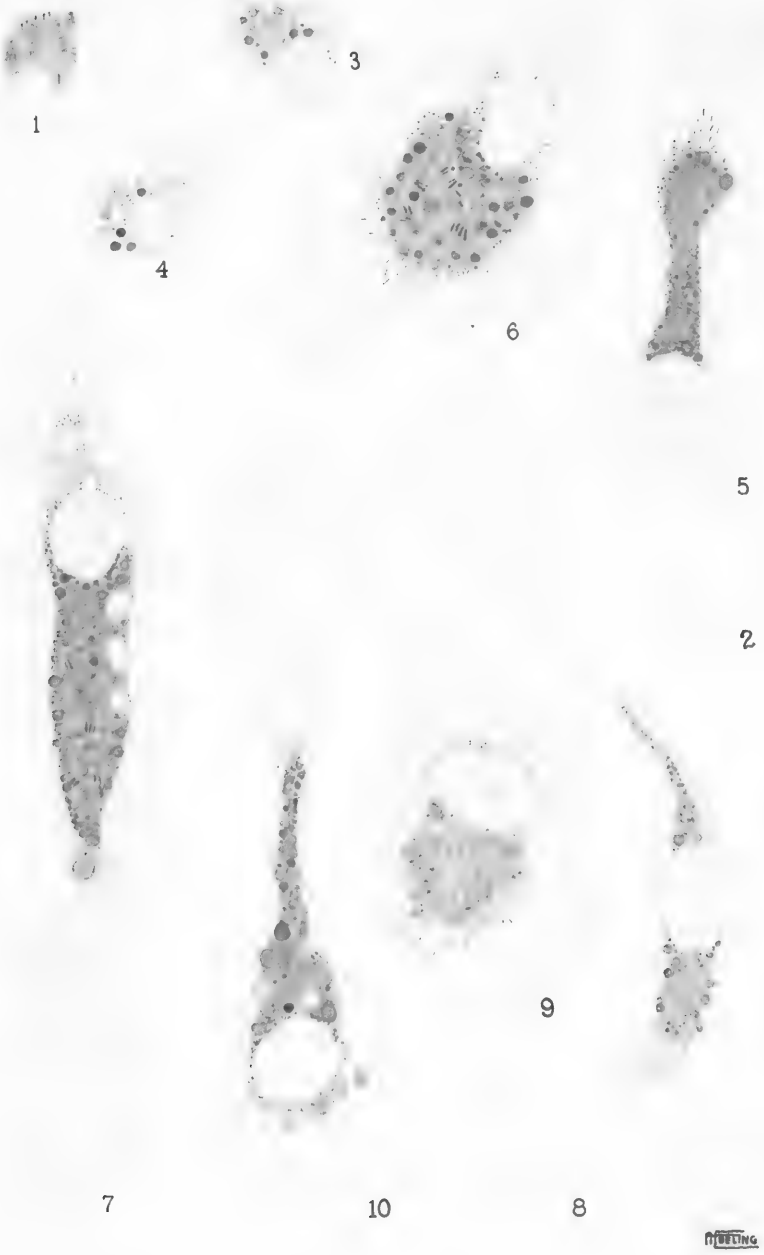
FIG. 14. Culture 3445-C2. Blood monocyte cultivated in serum for 4 days.

FIG. 15. Culture 3471-C1. Blood monocyte cultivated in Ringer solution for 3 days.

FIG. 16. Culture 3471-C2. Blood monocyte cultivated in serum for 3 days.

FIG. 17. Culture 3841-C1. Macrophage from subcutaneous tissue of an adult chicken after 24 hours cultivation in plasma and embryo juice.

FIG. 18. Culture 3841-C2. Macrophage from subcutaneous tissue of an adult chicken after 5 days cultivation in Tyrode solution.



(Carrel and Ebeling: Properties of fibroblast and macrophage. II.)

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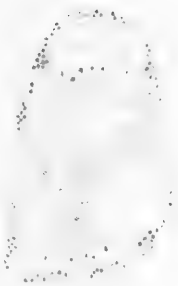

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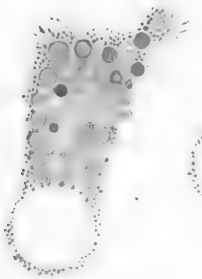

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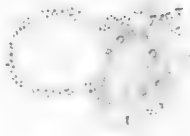

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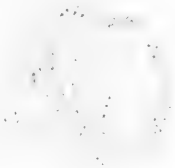

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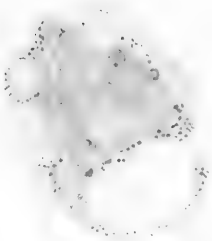

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(Carrel and Ebeling: Properties of fibroblast and macrophage. 11.)

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COMPARATIVE STUDIES OF HERPETOMONADS AND LEISHMANIAS.

I. Cultivation of Herpetomonads from Insects and Plants.

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Plates 10 to 15.

(Received for publication, March 16, 1926.)

Our interest in the herpetomonads parasitic in insects and plants in various parts of the world lies in the possible relationship of some of them to the parasites of kala-azar, of India, China, and Mediterranean countries, oriental sore, of Palestine, and espundia, of South America. The mode of infection of man by the leishmanias is not understood, and experimental work on the problem of transmission has, until recently,[1] been rendered extremely difficult by the lack of susceptible laboratory animals. The geographic distribution and epidemiology of the various forms of leishmaniasis have indicated that the parasites may be carried by some insect, and many attempts have been made to find evidence of this mode of transmission.

That the leishmanias may be taken up by insects and are capable of surviving in the insect gut was first shown in the case of *Leishmania donovani* by Patton,[2] who fed bedbugs on kala-azar patients and observed the development of the parasites to the flagellate stage in the bugs. Wenyon[3] later reported the ingestion of *Leishmania tropica* from open lesions of cutaneous leishmaniasis by flies, mosquitoes, and bedbugs, and the development of the parasites in some of these insects. The sand fly (*Phlebotomus*) is looked upon at present[4] as the most probable vector of leishmaniasis, but the experimental evidence is fragmentary.

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[1] Young, C. W., and Liu, Pao-Yung, *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 392.

[2] Patton, W. S., *Scient. Mem. Off. Med. and San. Dept. Gov. India, No. 27*, 1907.

[3] Wenyon, C. M., *Parasitology*, 1911, iv, 273.

[4] Sinton, J. A., *Indian J. Med. Research*, 1924–25, xii, 701.

The search for pathogenic varieties of herpetomonads among the flagellates so commonly found inhabiting the intestinal tract of insects was begun in 1913 by Laveran and Franchini, who reported having induced in rats and mice a disease not unlike kala-azar or oriental sore by inoculation of flagellates obtained from the dog flea, mosquito, sheep ked, and house fly, and even of flagellates of plant origin.[5] Pure cultures were used for inoculation in most instances. Later they obtained somewhat similar results with cultures of a flagellate obtained from the sand fly.[6] Fantham and Porter[7] induced symptoms of kala-azar by inoculation of various insect flagellates and believed that the leishmaniases would prove to be insect-borne herpetomoniases, but Hoare[8] repeated their experiments and obtained only negative results. Becker[9] and Drbohlav[10] also failed to infect animals with flagellates of insect origin. Strong,[11] who found flagellate infections in the hemipteran, *Chariesterus cuspidatus*, of Panama, in its plant hosts, and in a lizard presumed to prey upon the insect, was able to cause ulceration of the skin in a monkey by inoculation of the flagellate material from the intestinal tract of the lizard and to demonstrate leishmaniform parasites in the skin lesion; but he found the plant and insect flagellates to be non-infective.

While a very large number of the plant and insect flagellates have been described, the greater part have been studied only under natural conditions and on the basis of morphological features, and they have usually been given specific names designating their hosts. It has been recognized, however, that the same flagellate may live in several hosts, and Becker,[9] having demonstrated by cross-infection experiments that the flagellates of *Musca domestica* could thrive equally well in the intestinal tract of several other species of flies, and that the flagellates inhabiting each of the fly species which he studied showed a similar adaptability, concluded that the herpetomonads of flies all represent the same species. Drbohlav[10] confirmed Becker's experiments with fly flagellates, using a cultivated strain of *Herpetomonas muscarum*, but was unable to infect the dog flea or water strider with any flagellate save the one naturally occurring in these insects. He was unsuccessful in establishing *Leishmania infantum* in any flagellate host studied.

A number of the flagellates of invertebrates and plants have been cultivated, among them *Herpetomonas culicis* from *Culex pipiens* (Novy, MacNeal,

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[5] Laveran, A., and Franchini, G., *Compt. rend. Acad.*, 1913, clvii, 744; *Bull. Soc. path. exot.*, 1914, vii, 665; 1919, xii, 379; 1920, xiii, 796.

[6] Laveran, A., and Franchini, G., *Bull. Soc. path. exot.*, 1920, xiii, 569.

[7] Fantham, H. B., and Porter, A., *Proc. Cambridge Phil. Soc.*, 1915, xviii, 39.

[8] Hoare, C. A., *Parasitology*, 1921, xiii, 67.

[9] Becker, E. R., *J. Parasitol.*, 1923, x, 25.

[10] Drbohlav, J. J., *Am. J. Hyg.*, 1925, v, 580, 599.

[11] Strong, R. P., *Am. J. Trop. Med.*, 1924, iv, 1.

and Torrey[12]), *H. ctenocephali* of the dog flea (Tyzzer and Walker,[13] Wenyon,[14] Laveran and Franchini[5]), *H. pulicis* from *Pulex irritans* (Wenyon[15]), *H. ctenopsyllæ*[5] of the mouse flea, *H. phlebotomi*[6] of the sand fly, *H. jaculum*[5] of the scorpion, *Crithidia melophagi*[5] of the sheep ked, and *H. tarantolæ*[16] of the gecko (Laveran and Franchini). Laveran and Franchini obtained pure cultures also from plants.[17] Nöller[18] cultivated a number of insect herpetomonads and several trypanosomes. Drbohlav[10] obtained pure line strains of *H. muscarum*. The medium used has usually been the N. N. N. medium or some modification of it, though Franchini and Laveran made use also of blood bouillon,[16] and Franchini[19] later found that a number of flagellates would grow in a mixture of bouillon and plant latex.

The purpose of the present investigation was to cultivate a number of herpetomonads from different sources and compare their cultural and biological properties. Serological comparison of the leishmanias[20] had already indicated the value of immunological properties for differentiation and had suggested that these and other biological properties might be used for comparative studies of the leishmanias with other herpetomonads. We have been able to supplement this study by comparison of our strains with other cultivated flagellates, *H. ctenocephali*, isolated by Tyzzer and Walker in 1919,[13] and *T. rotatorium*, isolated by Drbohlav,[10] cultures of which were furnished us through the courtesy of Dr. E. E. Tyzzer, of Harvard University Medical School.

Pure cultures of herpetomonads have been isolated from the following insects and plants: *Oncopeltus fasciatus* (Fig. 1), *Oncopeltus sp.?*[21] (Fig. 2), *Lygæus kalmii* (Fig. 3) (two strains), *Anopheles quadrimacula-*

[12] Novy, F. G., MacNeal, W. J., and Torrey, H. N., *J. Infect. Dis.*, 1907, iv, 223.

[13] Tyzzer, E. E., and Walker, E. L., *J. Med. Research.*, 1919, xl, 129.

[14] Wenyon, C. M., *Tr. Soc. Trop. Med. and Hyg.*, 1914, vii, 104.

[15] Wenyon, C. M., *Arch. Protistenk.*, 1913, xxxi, 1.

[16] Laveran, A., and Franchini, G., *Bull. Soc. path. exot.*, 1921, xiv, 323.

[17] Laveran, A., and Franchini, G., *Bull. Soc. path. exot.*, 1920, xiii, 796.

[18] Nöller, W., *Arch. Schiffs- u. Tropen-Hyg.*, 1917, xxi, 53.

[19] Franchini, G., *Bull. Soc. path. exot.*, 1923, xvi, 41.

[20] Noguchi, H., *Proc. Internat. Conf. Health Prob. Trop. America*, Kingston, Jamaica, British West Indies, July 22 to August 1, 1924, published in Boston, 1924, p. 455.

[21] Specimens of this insect have recently been submitted for identification to Dr. H. G. Barber of the American Museum of Natural History, who was able at once to classify it as to genus. The species has not yet been determined.

tus, *Culex pipiens*, *Musca domestica*, bluebottle flies (two strains), *Asclepias syriaca* (two strains), and *Asclepias nivea*. In addition, two impure cultures were obtained, one from *Oncopeltus cingulifer* (Fig. 4), and one from the plant on which it feeds, *Asclepias curassavica*. Attempts to purify these two strains were unsuccessful, and they soon died out.

The specimens of *O. cingulifer* were collected in Tela, Honduras,[20] those of *Oncopeltus sp.?* in the vicinity of Lima, Peru,[22] those of *O. fasciatus* and *L. kalmii* on Long Island and in Shandaken, N. Y., those of *Anopheles quadrimaculatus* in Leesburg, Georgia,[23] and those of *Culex pipiens* near Jersey City. The house and bluebottle flies were collected on the grounds of The Rockefeller Institute. The specimens of *A. curassavica* were brought from Tela,[20] Honduras, and those of *A. syriaca* from Baltimore, Long Island, and Shandaken. For the Haitian milkweeds, *A. nivea*, we are indebted to Dr. Francis O. Holmes, of the Boyce Thompson Institute for Plant Research, Yonkers, N. Y., who infected them by allowing infected specimens of *Oncopeltus fasciatus* to feed upon the seed pods.

For identification of the latex-feeding insects we are indebted to Dr. H. G. Barber, of the American Museum of Natural History. The bluebottle flies were no doubt *Calliphora*, but identification of the species was not possible in these instances.

Method of Cultivation.

For the initial cultivation of the flagellates from insects, solid blood agar plates were employed which contained various carbohydrates and were adjusted to different hydrogen ion concentrations, pH 5, 5.5, 6, 6.5, 7, and 7.5. The proportions of the constituents were as follows:

Defibrinated horse or rabbit blood		300 cc.
2 per cent nutrient agar, melted		500 cc.
Mixed sugar solution, sterilized by filtration through Berkefeld filters		25 cc.
Glucose	20 gm.	
Saccharose	10 "	
Inulin	5 "	

[22] These specimens were collected by Dr. T. S. Battistini, of Lima.

[23] Dr. Mark F. Boyd, of the International Health Board, was kind enough to inform us of the presence of flagellates in these mosquitoes and also to dissect insects and inoculate culture media.

Levulose	5 gm.
Raffinose	5 "
Distilled water	100 cc.
0.9 per cent sodium chloride solution	175 cc.

Various bacteria, molds, and yeasts are usually associated with the flagellates in the insect gut and by their more rapid growth render the isolation of the latter extremely difficult. In several instances impure cultures were obtained by inoculating plates with ascending dilutions of the suspension of teased viscera, and these were purified by transfers to new plates repeatedly until some pure colonies of flagellates appeared. Later, however, in the work with flies, it was found necessary to resort to Barber's technique[24] of picking out one flagellate or a group of them for inoculation of the plates. Theoretically this procedure ought to give fairly constant results, but actually many plates implanted with a single flagellate failed to show any growth, though plates spread gently with several organisms usually yielded some isolated colonies. Probably not every individual was capable of adapting itself to the cultural conditions provided.

It was noticed that whenever an impure culture was obtained by the plate method, the contaminating organisms were usually yeasts, which had apparently suppressed the growth of bacteria to some extent by the production of acidity. The observation suggested the use of culture media adjusted to concentrations of acid which were not sufficient to interfere with the growth of the flagellates, but in which the growth of bacteria was reduced so far as possible. The neutral and slightly alkaline media were not omitted in routine work, however. As a rule, four or five plates of a given hydrogen ion concentration were used; a small drop of the suspension of teased viscera in 0.5 per cent saline was spread on the first plate with a sterile bent pipette, and the other plates were brushed successively with the same pipette. The first plate was usually overgrown by bacteria, but one of the others might yield fairly discrete colonies of bacteria and an occasional pure colony of flagellates.

The cultivation of plant flagellates was much more difficult, not because of the contaminants—for in this instance the only contaminants present are the yeasts and molds from the stem or leaves—but because of the difficulty of adaptation of the flagellates to the media, as shown by the fact that when large numbers of them were introduced they usually lost their motility within a few days and finally degenerated. Pure cultures have thus far been obtained only on leptospira medium,[25] and only three times among many attempts. In one other in-

[24] Barber, M. A., *Philippine J. Sc., Section B*, 1914, ix 307.

[25] The formula is as follows: 0.9 per cent NaCl, 800 parts; fresh rabbit serum, 100 parts; 2 per cent nutrient agar (pH 6.5 to 7.0), 100 parts; rabbit hemoglobin (made by laking 1 part of defibrinated blood with 3 parts of distilled water), 10 parts.

stance an impure culture of flagellates from *A. curassavica* was obtained on a medium composed of the usual leptospira medium plus glucose, inulin, and tapioca.

The isolation experiments were carried out at room temperature, which varied from 18°C. to 28°C. during the day and was probably somewhat lower at night. All the plates were sealed with adhesive tape and kept in a moist chamber. Examinations were not made until after 7 to 10 days, experience having soon shown that there was no advantage in earlier examination. The colonies of flagellates develop very slowly, and they are so minute that very close examination is necessary to find them. In initial cultures 10 to 14 days were required in most instances for the colonies to become visible as tiny dew-like spots. When associated with bacteria, the flagellates can only be detected by microscopic examination, and for this purpose the dark-field microscope offers the most convenient and rapid means of detection.

From the pure colonies of flagellates found on plates with well separated colonies, subcultures were readily obtained on similar media (plates or slants), and still more certainly on leptospira medium, with or without carbohydrates. On this medium growth is remarkably rapid and prolific, in marked contrast to that of the first generation. All the strains isolated during the present experiments grow much more rapidly and profusely than any of the leishmanias or the strains of flagellates from dog flea or frog. They grow well at 37°C., as well as 25°C., while for the other flagellates the lower temperature is more suitable.

Description of Flagellates Studied.

Seven of the twelve strains isolated were derived from milkweed-feeding insects and their plant hosts. When the cultures came to be studied with respect to their serological and carbohydrate-fermenting properties, it was found that the seven strains represented in reality only two species of flagellates. Similarly, the two strains from mosquitoes behaved as one serologically. If we were to classify the strains according to host species, we should have to create several names for organisms which by morphology and biological reactions show identical characteristics, while if we were to depend on morphology alone, a number of flagellates which behave differently would have to be classed as a single species. The study of biological relationships, however, made it possible to identify a given flagellate in different environments and to separate different flagellates occurring in the same host. The classification of the strains was accordingly very much simplified. In order to avoid confusion it seems desirable to employ throughout this paper the nomenclature finally adopted,

TABLE I.

Species.	Source.	Morphology in insect or plant.	Morphology in culture on leptospira medium.
H. oncopelti.	*Oncopeltus fasciatus.* *Oncopeltus sp.?* *Lygæus kalmii* No. 1.	Narrow, slender bodies; anterior end rounded, posterior pointed. Flagellum about the length of the body.	Similar to insect forms, but usually smaller, with shorter flagellum. Anterior end sometimes truncated.
	Asclepias syriaca No. 213. *Aselepias nivea.*	Characteristic ribbon-like bodies, sometimes twisted; flagellum usually short.	
H. lygæorum.	*Lygæus kalmii* No. 2.	Slightly longer and narrower than flagellates of *L. kalmii* No. 1; flagellum somewhat longer.	
	Asclepias syriaca No. 46.	Characteristic ribbon-like forms, but somewhat shorter than those of *A. syriaca* No. 213.	
H. culicidarum.	*Anopheles quadrimaculatus* *Culex pipiens.*	Short, truncated forms, with short flagellum.	Similar to forms in insect, but considerably larger.
H. muscidarum.	*Musca domestica.*	Large forms, many with characteristic long double flagellum.	Similar to *H. oncopelti* in young cultures; much larger in old cultures, but never as large as *H. muscæ domesticæ* of von Prowazek.
H. media.	*Calliphora sp.?* No. 1.	Smaller than flagellates of *M. domestica;* flagellum long.	Similar to young cultures of *H. muscidarum*, but smaller and narrower; never become large.
H. parva.	*Calliphora sp.?* No. 2.	Similar to *H. media.*	Small, rounded forms, with very short flagellum.

although the manner in which it was arrived at is chiefly discussed in Part II of our report.[26]

Table I is a résumé of the names, source, and morphology of all the flagellates grown in pure culture. Identical strains from two or more sources have been named in accordance with the source from which they were first cultivated (*H. oncopelti*, *H. lygæorum*).[27] The mosquito flagellates, which proved identical, have been called *Herpetomonas culicidarum*, since the same strain was isolated from two genera of the family *Culicidæ*. As will be clear from the experiments described in Part II of this report, it was not possible to identify this organism with others previously described as occurring in mosquitoes.

The naming of the fly strains was a more difficult problem, inasmuch as each of the three proved to be distinct from the others. The preparations of the flagellates found in the gut of *Musca domestica* show chiefly large forms with double flagellum which conform with the descriptions of *H. muscæ domesticæ*, but the organisms seen in young cultures are not unlike *H. oncopelti* and *H. lygæorum*. As the cultures grow older much larger forms appear, but these do not correspond exactly with the naturally occurring individuals, and it is difficult to exclude the possibility that some other strain than *H. muscæ domesticæ* was simultaneously present in the insect and grew more readily in culture. It was likewise impossible to identify two distinct strains from *Calliphora* as *H. calliphoræ*. The strains were finally given the new species designations, *Herpetomonas muscidarum*, *H. media*, and *H. parva*.

All the flagellates cultivated have a single anterior flagellum, arising near the parabasal body, which is situated beside, or in front of, the nucleus. No undulating membrane, such as characterizes *Crithidia* and *Trypanosoma*, has been demonstrated in any of the strains, either under natural conditions or in culture. The term *Leptomonas*, while it has priority over *Herpetomonas*, is less well defined, and it seems reasonable, as suggested by Wenyon,[15] to restrict the name to the species for which it was created until it can be proved that *Herpetomonas* is actually a synonym of it. Werner's objection to *Herpeto-*

[26] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 327.

[27] The name *H. lygæi* has been used by Patton (*Arch. Protistenk.*, 1909, xiii, 1) to designate a flagellate species found in *Lygæus militaris* of India.

monas,[28] on the ground that it was created for a biflagellate genus, has been invalidated by Wenyon's cytological study[15] of *H. muscæ domesticæ*, which demonstrates that the biflagellated forms are actually individuals in a very early stage of division.

Herpetomonas oncopelti, n. sp. (*Figs. 5, 6, 9, 10, 13, 14, 17, 18*).

Strain 1.—This strain (Fig. 5) was obtained from the intestinal tract of *Oncopeltus fasciatus* (Fig. 1) caught in Shandaken, N. Y. The insect feeds on the latex of the common milkweed, *Asclepias syriaca*, which is of widespread occurrence in the temperate regions of the United States. According to Holmes,[29] the insect is rarely found north of New Jersey, being replaced in the northeastern states by the species *Lygæus kalmii*, which also feeds on the latex of *A. syriaca*. Only three specimens of *O. fasciatus* have been obtained in the course of the present study (one on Long Island, and two in Shandaken), and only one of these was infected with herpetomonads. Farther south, however, in New Jersey and Maryland, Holmes found that the majority of specimens harbored flagellates.

Morphology in Insect Host.—The forms found in the insect (Figs. 5, 9, 17) have relatively narrow bodies, the anterior end is somewhat rounded, and the body gradually narrows to a point at the posterior end. The flagellum originates near the parabasal body, which is round or oval and is situated closer to the anterior end than to the nucleus. In most specimens the flagella are about the length of the body, perhaps longer. When the organisms are moving swiftly in forward motion, the flagellum vibrates rapidly; in less active specimens it has an undulating, serpentine motion and may sometimes be alternately coiled into loops and stretched out. None of the flattened or twisted ribbon-like bodies so characteristic of plant herpetomonads has been encountered. Occasionally short, broad, pear-shaped forms have been observed, some of which have long and others very short flagella.

Cultural Properties.—Once obtained in culture, the organism is easily grown on the semisolid leptospira medium or on any of the blood agar slants, such as the N.N.N. or Nöller's medium, which have been widely employed for the cultivation of leishmanias and insect flagellates. On leptospira medium the growth becomes visible within 48

[28] Werner, H., *Arch. Protistenk.*, 1909, xiii, 19.
[29] Holmes, F. O., *Biol. Bull.*, 1925, xlix, 323.

hours at room temperature as a grayish white, usually homogeneous mass, which gradually extends deeper into the medium. At 37°C. growth can be recognized macroscopically after 24 hours. On blood agar slants grayish, dew-like, moist and shining colonies become visible within 72 hours at room temperature and 48 hours at 37°C. They gradually spread and become confluent, and the layer of growth assumes a light grayish color. The colonies are of a sticky consistency, and a thin grayish pellicle may form on the surface of the condensation water.

The morphological features vary greatly according to the medium on which the organism is grown. In young colonies on the surface of blood agar slants (Figs. 6,*a*, 10,*a*) most of the individuals are pear-shaped, oval, or truncated at the anterior end, with a short flagellum which moves constantly from side to side. The flagellum arises far in the interior of the cell, the parabasal body lying at one side of the nucleus. After some days the organisms become aggregated into rosettes of varying size, the flagella being directed inward, the bodies becoming uniformly narrower, pyriform, or spindle-shaped, and the flagella longer. In the condensation water much longer, slenderer forms are found, with flagella as long as the body.

The flagellates grown on leptospira medium are of elongate form, with flagella usually the length of the body (Figs. 6,*b*, 10,*b*). Unusually long forms are found in cultures which have been kept at room temperature more than 2 or 3 weeks, and these resemble the specimens of the organisms in the insect host. The parabasal body occupies a position anterior to the nucleus, the anterior portion of the organism is wide and rounded, and the body narrows down to a slender, pointed posterior portion. These long forms with well developed flagella travel about freely and swiftly in all directions, in contrast to the short truncated forms, with vibrating flagella, which rotate swiftly but do not travel out of the microscopic field.

Numerous bizarre forms appear in media having a pH near 5. In some the cytoplasm is divided except at the anterior portion, where a single flagellum arises, the division beginning near the anterior portion or at any point farther toward the posterior end. There may be one, two, or several clefts in a single organism, and the lobes are usually unequal in size. It is difficult to interpret this phenomenon, which

has also been observed under natural conditions in the insect host, but it appears to be an abnormal multiple division.

Strain 2 (*from Oncopeltus sp.?*).—Figs. 9, 10. Another species of *Oncopeltus* (Fig. 2) was obtained by Dr. Telémaco S. Battistini in the vicinity of Lima. Its plant host is also a species of *Asclepias*. (The specimens of milkweeds which Dr. Battistini collected, however, proved to be free from flagellates.) The initial impure culture of this flagellate was purified by the Barber method. No morphological differences could be detected between this strain and the one derived from *Oncopeltus fasciatus*.

Strain 3 (*from Lygæus kalmii No. 1*).—Figs. 17, 18. *Lygæus kalmii* (Fig. 3) resembles *O. fasciatus* in appearance, and its geographic distribution is somewhat similar. In the Catskill Mountains (Shandaken), at an elevation of 1000 feet above sea level, *Lygæus kalmii* far exceeded *O. fasciatus* in number. Four of the twenty-four specimens collected harbored flagellates, and in two instances cultivation was successful. While the two strains were morphologically indistinguishable in culture, they proved subsequently to be distinct species, one of which is identical with *H. oncopelti*.

Strain 4 (*from Asclepias syriaca No. 213*).—The occurrence of herpetomonads in *Asclepias syriaca* (Fig. 30) was first reported by Holmes,[30] who regarded the organisms found by him as identical with *H. elmassiani* of Migone, described by França.[31] Of two strains of flagellates isolated from two infected milkweeds collected in Shandaken, N. Y., one proved to be *H. oncopelti*. The initial growth was obtained on leptospira medium and was not detected until several weeks after inoculation. The culture was purified by plating.

Strain 5 (*from Asclepias nivea*).—Figs. 13, 14. Through the kindness of Dr. Francis O. Holmes, of the Boyce Thompson Institute for Plant Research, a plant of the species *Asclepias nivea*, a Haitian milkweed, was placed at our disposal. It had been infected by allowing infected specimens of *Oncopeltus fasciatus* to feed on the seed pods. The morphology of these flagellates in the latex (Fig. 13) is similar to that of *H. oncopelti* in *Asclepias syriaca* (Fig. 30). They are characteristically ribbon-like, with twisted bodies, clear, almost hyaline protoplasm, and short flagellum. In culture (Fig. 14), however, they cannot be distinguished from the culture forms derived from hemipterans. The initial culture was pure, and, as in the case of all the plant strains, was obtained on leptospira medium. Only one tube of twelve inoculated yielded growth.

Herpetomonas lygæorum, n. sp. (*Figs. 11, 12, 19, 20*).

Strain 1 (*from Lygæus kalmii No. 2*).—As has already been mentioned, cultures of herpetomonads were obtained from two insects of the species *Lygæus*

[30] Holmes, F. O., *Phytopathology*, 1924, xiv, 146.

[31] França, C., *Ann. Soc. belge med. trop.*, 1920–21, i, 245.

kalmii. The preparations made from suspensions of the intestinal tract show that the forms in *Lygæus* No. 2 were a trifle narrower and longer than the majority of those in *Lygæus* No. 1, and had somewhat longer flagella. The morphological characteristics of the two cultures, however, are very much the same (Figs. 18, 20). The two species can be distinguished only by serological and fermentation reactions.

Strain 2 (*from Asclepias syriaca No. 46*).—Figs. 11, 12. The impure culture of this strain was discovered several weeks after inoculation of leptospira medium with the plant latex. Purification was accomplished by the Barber technique. In the plant (Fig. 11) and also in culture (Fig. 12), this organism is indistinguishable from the strain from *A. syriaca* No. 213, except for slight difference in size, but serologically and in fermenting properties it is identical with *H. lygæorum*.

Herpetomonas sp.? from Oncopeltus cingulifer (*Figs. 7, 8, 33, 34*).

Oncopeltus cingulifer (Fig. 4) feeds on the latex of *Asclepias curassavica*, a Honduran milkweed, in which flagellates were first found by Hegner.[21] The relation between the insect strain and that of its plant host could not be ascertained in this instance, since pure cultures were not obtained. The impure growth was detected 75 days after inoculation on one of several dozen plates which had been similarly inoculated and kept under identical conditions. The flagellates of *O. cingulifer* differed in appearance from *H. oncopelti* in having a considerably longer flagellum.

Herpetomonas sp.? from Asclepias curassavica (*Figs. 15, 16, 31, 32*).

An impure culture of this organism was obtained on a semisolid (leptospira) medium containing glucose, inulin, and tapioca. The flagellates in the culture (Figs. 16, 32) bore rather slight resemblance to the forms found in the latex (Figs. 15, 31) and were much longer than those of the pure cultures isolated subsequently from American and Haitian milkweeds (Figs. 12, 14). Their morphological features do not correspond with those of the cultural forms (impure) of *H. elmassiani* obtained by Migone from *Aranjia angustifolia* in Paraguay, as described by França.[31]

Herpetomonas culicidarum, n. sp. (*Figs. 21, 22*).

Through the cooperation of Dr. Mark F. Boyd, of Leesburg, Georgia,[23] we were able to secure an impure culture of a *Herpetomonas* from *Anopheles quadrimaculatus* on a blood sugar slant (pH 5). The strain was purified by plating on similar medium. The original film preparation made by Dr. Boyd (Fig. 21) contained numerous flagellates of truncated and spindle shape, with short flagella. The flagellates in culture (Fig. 22) were considerably longer, in this instance, than those in the original material. In all other instances the reverse was true.

Strain from Culex pipiens.—Small numbers of herpetomonads were found in the intestines of two larvæ of *Culex pipiens*, collected in New Jersey, and from one of them a culture was obtained on acid blood agar plates. The immunological and fermenting properties of this strain are identical with those of the flagellate from *A. quadrimaculatus*.

Herpetomonas muscidarum, n. sp. (from Musca domestica) (Figs. 23, 24).

Many of the house flies collected about the grounds of The Rockefeller Institute harbored herpetomonads, some of them very large forms with long active flagellum, in many instances double, as described by von Prowazek.[32] A section of intestinal tract from one of the infected flies, in which the flagellates were swarming as in culture, was picked out intact with a fine pipette, washed, and the contents suspended in 0.5 per cent saline. Inoculation of acid blood agar with ascending dilutions of this suspension yielded some pure colonies.

While the cultural properties of this organism are very much the same as those of the other cultivated strains of the series, in morphology it is slightly different. In young cultures on leptospira medium (Fig. 24, *a*) the individuals are no larger and the flagella no longer than in the case of *H. oncopelti* and *H. lygæorum*, but after several weeks in this medium extremely long forms appear, with flagella not unlike those of the organisms in the original material (Fig. 24, *b*).

Herpetomonas media, n. sp. (from Calliphora No. 1) (Figs. 25, 26).

This culture, which was obtained by the Barber method, is a pure line strain. Morphologically this organism resembles *H. muscidarum*, but it never attains the size of that strain, even in old cultures, and it is immunologically distinct.

Herpetomonas parva, n. sp. (from Calliphora No. 2) (Figs. 27, 28).

This culture also represents a pure line strain. It is decidedly smaller than the other two strains from flies. On blood agar slants the colonies of *media* and *parva* are light gray, heavier than those of *muscidarum*, and show less tendency to coalesce.

Herpetomonas ctenocephali; Trypanosoma rotatorium; the Leishmanias.

Morphologically, *H. ctenocephali* is quite unlike any of our strains. In its leaf-like form it resembles rather the flagellates found in the plant latices, though the flagellum is much longer. The frog trypanosome is again entirely different from any of the other flagellates studied. On the other hand, the morphological features of the leishmanias, while distinctive, approach those of the culture

[32] von Prowazek, S., *Arb. k. Gsndhtsamte*, 1904, xx, 440.

TABLE II.

Source of flagellate.	Length of body.	Anterior end to parabasal body.	Parabasal body to nucleus.	Nucleus.	Posterior end to nucleus.	Width at widest portion.	Length of flagellum.	Remarks.
	μ	μ	μ	μ	μ	μ	μ	
Oncopeltus fasciatus	12–25	1.8–3.5	2–3	2.5–3	12–15	2–3	10–25	Shandaken.
" " (culture forms)	11.5–17	2–4	2–4	2.5–3	1.2–6	2–3.5	6–18	
Oncopeltus cingulifer	12–25	2–4	0.5–3	1.8–2.8	6–15	2–3	10–28	Honduras.
" " (culture forms)	7–16.5	1.5–3.5	2.3–4.5	1.5–2.8	0.3–3.5	1.8–8	4–18	"
Oncopeltus sp.? (culture forms)	7–16.5	1.5–3.5	2.3–4.5	1.5–2.8	0.3–3.5	1.8–5	4–18	Peru.
Lygæus kalmii	8–12	1.8–2	1–1.5	1.8–2	5–8	1.8–2.5	6–12	Shandaken.
" " (culture forms)	7–14	1.8–3.5	0.5–3	1.8–2.2	2–6	2.5–3.5	6–16	Strain 1.
" " " "	6–12	2–3	0–2 (some beside nucleus).	1.8–2.2	3–4	2–2.5	4–14	" 2.
Anopheles quadrimaculatus	6–8	1.8–2	0.5–1	1.8–2	2–2.8	1.8–2.5	4–6	Dr. Boyd's preparation.
" " (culture forms)	6–11	1.8–2	0.5–2.2	1.8–2	2–5	2–3	8–12	
Culex pipiens								Too few specimens in preparation. Similar to *Anopheles* forms.
" " (culture forms)	8–14	2–3	2–4	2–3	3–5	3–5	8–12	
Musca domestica	15–30	2.5–3	2–3	2.5–3	7–10	3–3.5	15–35	
" " (culture forms)	12–30	3–5	2–6	2.5–4	4–20	3–4	10–30	

Calliphora spl.	16–20	1.5–2	2–2.5	2–2.2	6–16	2.5–3	8–25	Strain 1.
" " (culture forms)	6–18	2–3	2–5	2–2.2	3–5	2.5–3	10–20	" 1.
" " " "	5–9	1.8–2	2–2.5	1.8–2.2	2–3	2–2.5	4–8	" 2.
Asclepias syriaca	8–12	1.8–2	1.8–2	2–2.2	4–6	2–2.5	2–10	Shandaken plant 46.
" "	13–22	1.5–2	1–2.5	2–3	5–14.5	2–3.5	6–12	" " 213.
" " (culture forms)	6–12	1.8–3	0.5–2.5	1.8–2.5	3–4	2–2.2	4–14	Strain 46.
" " " "	7–17	1.5–3.5	2.2–4	1.8–2.5	1–6	2.8–3.5	6–16	" 213.
Asclepias nivea	13–22	1.5–2	2–3	2–3	5–14.5	2–3.5	6–12	
" " (culture forms)	10–17	2–4	2–4	2.5–3	2–6	2.5–3.5	6–18	
Asclepias curassavica	14–20	1.2–2	1.3–4	2–3	8–14	2–4	3–12	Honduras.
" " (culture forms)	10–18	2–4	2–3	2–2.5	2–6	2.5–3	6–20	Impure culture.
Ctenocephalus canis.								
H. ctenocephali H.	Round, 3.3–6					2.5–4.3		After Tyzzer and Walker.[13]
	Kinetic, 11–16.7					1.3–2.5	7.3–21	
" " " (culture forms)	10–15	1–1.2	1–1.5	1.5–1.8	5–10	1.8–2	15–18	H strain. Twisted. Sharply pointed at both ends.
Rana pipiens.								
Trypanosoma rotatorium (culture forms)	10–15	1–1.2	1–1.5	1.5–1.8	5–10	1.8–2	6–8	Twisted and sharply tapered posterior portion.
Oriental sore.								
Leishmania tropica	10–12	1.5–2	1.8–2.2	1.8–2.2	6–8	2–2.5	10–15	Slender forms.
Espundia.								
L. brasiliensis	5–8	1.8–2	0.5–1	1.8–2.2	1–2.5	1.5–3	3–6	Stocky "
Kala-azar (China).								
L. infantum.	9–12.5	1–1.2	1–1.5	1.5–1.8	4–6	2–2.5	3–6	Sharply drawn to ends.

forms of our insect and plant flagellates; they are pyriform and stocky when grown on the surface of blood agar slants, but characteristically longer and slenderer on leptospira medium, and have longer flagella.

The flagellates of the dog flea and frog grow very slowly, 1 to 2 weeks being required for the growth on blood agar slants to become visible. The leishmanias grow somewhat more rapidly, but much more slowly than the plant and insect strains. All the strains studied show more rapid and prolific growth on leptospira medium than on plates or slants.

Table II is a comparison of certain morphological features of the flagellates studied. Preparations of cultures grown on leptospira medium were used in making the measurements of culture forms, except in the case of *Leishmania brasiliensis*; in this instance the stocky forms obtained from a blood slant culture were measured.

The preparations used as a basis of comparison were all made by fixing dried films in methyl alcohol and staining with Giemsa's solution. The early part of the work was not done under laboratory conditions, and this method was the only one which could be conveniently carried out. Later the cultures were studied by cytological methods (wet fixation in Schaudinn's sublimate alcohol, followed by staining with iron-hematoxylin or Giemsa's solution, with subsequent differentiation), but since these preparations furnished no additional information regarding the structure of the organisms and were not available in all instances, they have not been especially mentioned in the present study.

SUMMARY.

Nine strains of herpetomonads have been isolated in pure culture from eight varieties of insects, and three strains from two species of plants. Four of the cultures were derived from latex-feeding insects (*Oncopeltus fasciatus*, *Oncopeltus sp. ?*, *Lygæus kalmii*) and three from latex plants (*Asclepias syriaca*, *Asclepias nivea*); two from mosquitoes (*Culex pipiens* and *Anopheles quadrimaculatus*), one from the house fly (*Musca domestica*), and two from bluebottle flies. In addition impure cultures have been obtained from *Oncopeltus cingulifer* and from its plant host, *Asclepias curassavica*.

The flagellates cultivated, all of which belong to the genus *Herpetomonas*, have been identified chiefly by their biological relationships,

which will be described in detail in Part II of this report. The seven strains from latex-feeding insects and latex plants represent two distinct species, which have been designated *H. oncopelti* and *H. lygæorum*. The two strains from mosquitoes proved to be the same organism and have been called *Herpetomonas culicidarum*. The culture obtained from *Musca domestica* contained larger individuals than those of any other strain, and the organism is morphologically distinct from either of the *Calliphora* strains. None of the fly flagellates cultivated could be identified with the species *H. muscæ domesticæ* or *H. calliphoræ*, and hence they have been given new names, *Herpetomonas muscidarum*, *H. media*, and *H. parva*.

Blood agar plates were used for initial cultivation of the strains from insects and the semisolid leptospira medium for isolation of the plant flagellates. A number of the strains were purified by plating on acid blood agar, a procedure which reduces considerably the growth of bacterial contaminants. The Barber technique was utilized for isolation of the flagellates from flies, because of the very large number of bacteria found with them in these insects, and, in one or two instances, for the purification of impure cultures. Once they have been obtained in culture, all the strains grow well on leptospira medium, as well as on blood slants. Growth takes place both at 26°C. and at 37°.

The morphology of the organisms is considerably modified by cultivation. This is especially true of the plant flagellates. In the latex they have ribbon-like bodies, often twisted, and comparatively short flagella; the protoplasm is clear, almost hyaline. The flagellates seen in the gut and feces of insects are usually large, slender organisms, with flagella as long as or even longer than the body, which contains numerous volutin granules in the cytoplasm. In cultures under parallel conditions the flagellates from both these sources become shorter and thicker, the plant forms no longer appear flat and ribbon-like, and in general the organisms approach one another in morphological features. Even in the case of the least modified insect flagellates, *i.e.* those from flies, there is never exact correspondence between the natural and the cultivated forms.

The morphological features of the cultivated flagellates vary according to the medium on which the organisms are grown and the age

of the culture. The flagellates grown on the surface of blood slants are pyriform, with truncated anterior portion, and short flagellum; in the condensation water, however, the individuals are elongated and have long active flagella. On the leptospira medium the slender active forms with long flagella predominate. In the presence of fermentable carbohydrate, or in medium containing considerable acid, peculiar bifurcated or multifurcated individuals are seen. Similar forms have been seen under natural conditions. Cultures of *Leishmania* behave in the same way under the conditions described.

There is a striking difference in rapidity of growth between the organisms isolated by us and the leishmanias, *H. ctenocephali*, and *T. rotatorium*. While the stock cultures of the group first mentioned multiply rapidly at 37°C., growth becoming visible within 24 hours, the latter group grow scarcely at all at 37° and only slowly at 25°, 1 to 2 weeks being required for growth to become macroscopically demonstrable.

While the flagellum of the leishmanias, as also of *H. ctenocephali*, is long, serpentine in its movements, and heavy, having the appearance of being enveloped by a sheath throughout its entire length, that of the recently isolated strains is thin, less flexible, and without the sheath-like appearance. The only exceptions to this rule are the flagellates from *Musca domestica* and *Calliphora* No. 1, which have a long flagellum not unlike that of the leishmanias.

As the foregoing observations indicate, morphological differentiation of the flagellates studied, while not impossible, is subject to error by reason of the variations due to age and cultural conditions. The flagellates of the latex-feeding insects, the plants, the flies, and the mosquitoes can readily be distinguished from *Leishmania* by their rapid growth at 37°C., but their differentiation from one another is possible only by serological and fermentation reactions.

EXPLANATION OF PLATES.

PLATE 10.

FIGS. 1 to 4. Latex-feeding insects, natural size. Fig. 1, specimen of *Oncopeltus fasciatus* caught on Long Island. Fig. 2, *Oncopeltus sp.* from Lima, Pera. Fig. 3, *Lygæus kalmii*, specimen from Long Island. Fig. 4, *Oncopeltus cingulifer* from Honduras.

PLATES 11 TO 14.

FIGS. 5 to 28. Herpetomonads of insects and plants, as they appear under natural conditions and in culture. Drawn from preparations stained with Giemsa's solution after fixation by methyl alcohol. Magnification × 1200. The species name and source are indicated in each instance. *a*, forms from plant cultures. *b*, forms from cultures on leptospira medium.

PLATE 15.

Magnification × 1200.

FIG. 29. *Herpetomonas davidi*, original preparation made by Lafont,[33] presented by Professor Emile Brumpt. Giemsa's stain.

FIGS. 30 to 34. Drawings from preparations fixed in methyl alcohol and stained with Giemsa's solution. Fig. 30, flagellates from a specimen of *A. syriaca*, a plant of the group found by Holmes[30] in Maryland and studied by him. Fig. 31, flagellates from the latex of *Asclepias curassavica* (Honduras). Fig. 32, impure culture of flagellates from *Asclepias curassavica*. Fig. 33, flagellates from the gut of *Oncopeltus cingulifer*, which feeds on *Asclepias curassavica*. Fig. 34, impure culture of flagellates of *Oncopeltus cingulifer*.

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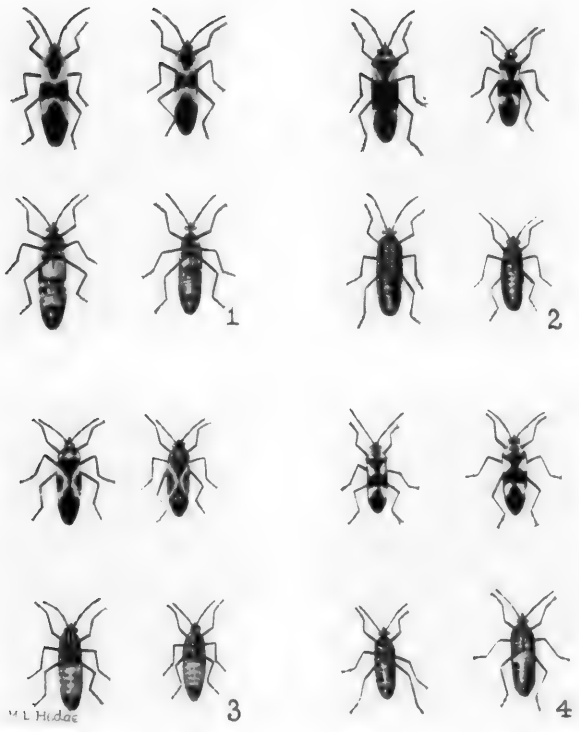
[33] Lafont, A., *Compt. rend. Soc. biol.*, 1909, lxvi, 1011.



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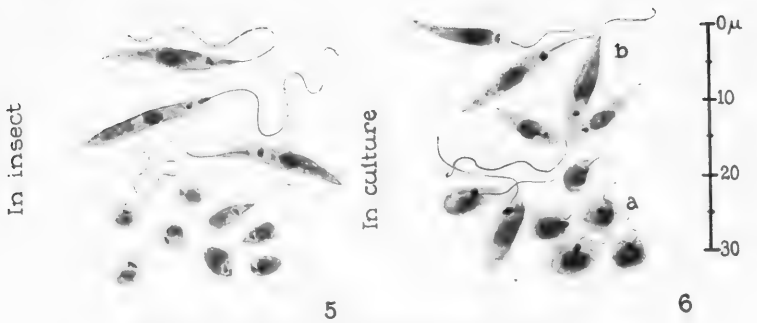
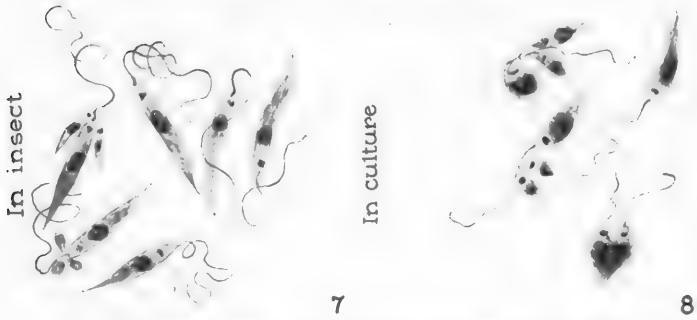


H.oncopelti (from Oncopeltus fasciatus)



Herpetomonas sp.? (from Oncopeltus cingulifer)

H.oncopelti (from Oncopeltus sp.?)

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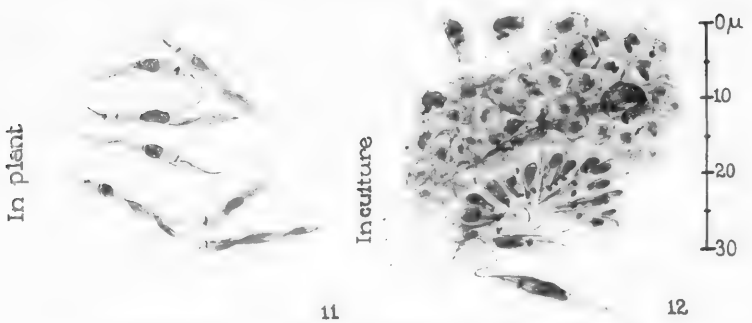
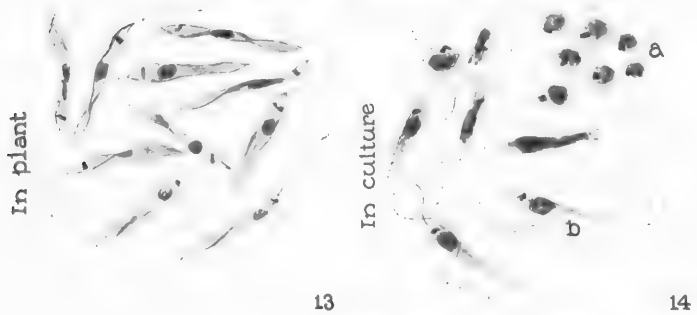
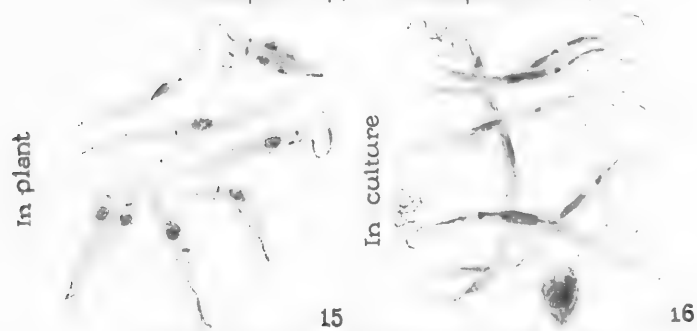


H. lygaeorum (from Asclepias syriaca No. 46)



H. oncopelti (from Asclepias nivea)



Herpetomonas sp.? (from A. curassavica)

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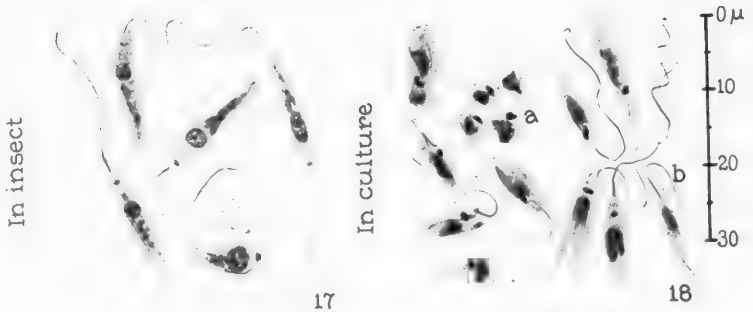
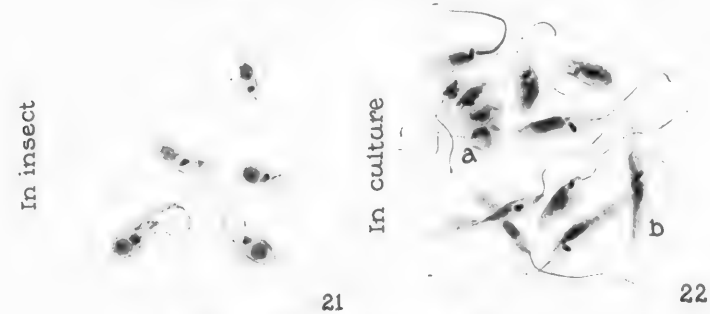


H. oncopelti (from Lygaeus kalmii No. 1)

H. lygaeorum (from Lygaeus kalmii No. 2)



H. culicidarum (from A. quadrimaculatus)

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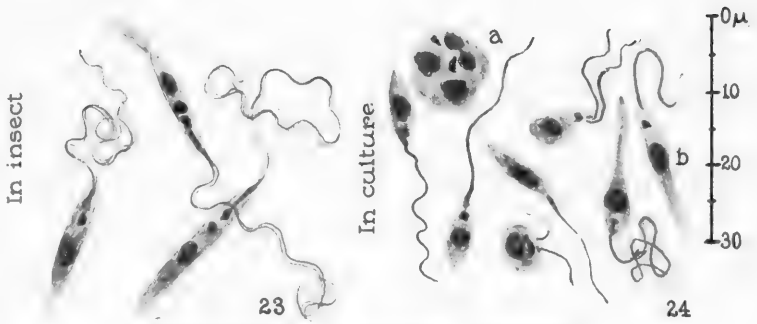
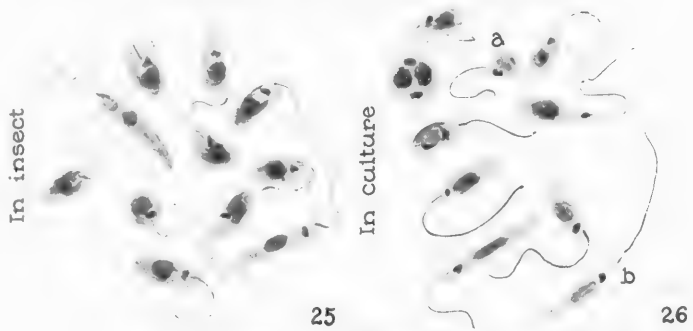


H. muscidarum (from Musca domestica)



H. media (from Calliphora? No. 1)

H. parva (from Calliphora? No. 2)

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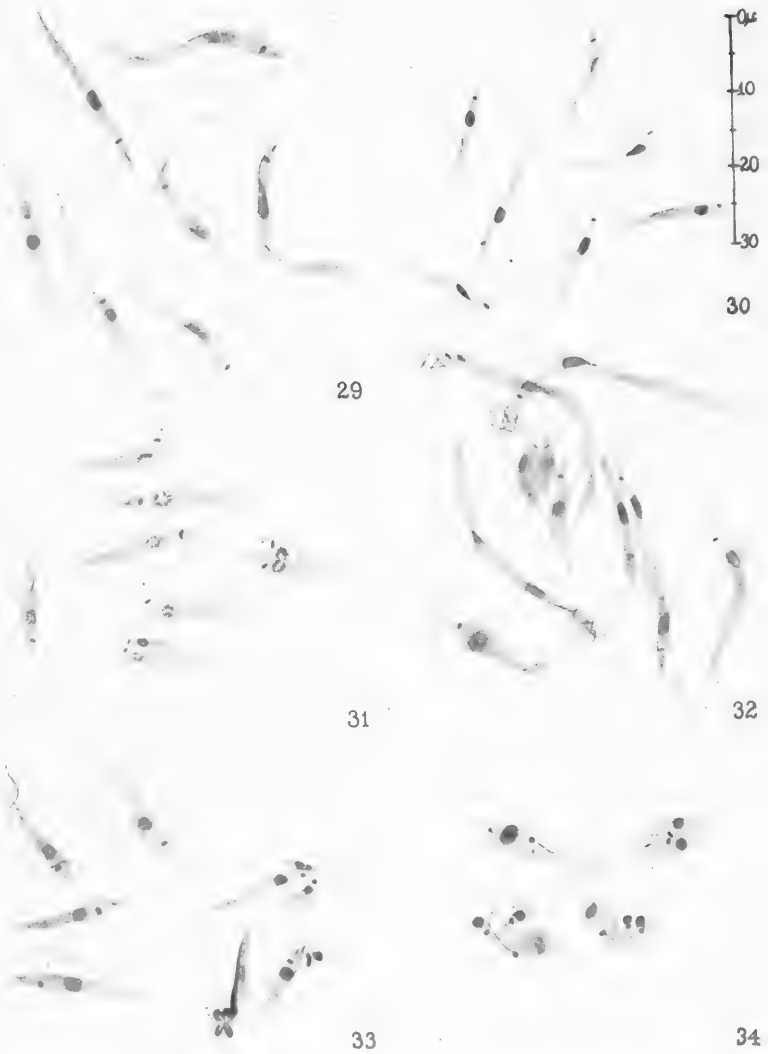

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COMPARATIVE STUDIES OF HERPETOMONADS AND LEISHMANIAS.

II. Differentiation of the Organisms by Serological Reactions and Fermentation Tests.

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(Received for publication, March 16, 1926.)

Rogers, who first cultivated the parasite of kala-azar[1] and found that under cultural conditions it acquired a flagellum and became morphologically indistinguishable from the herpetomonads so widely distributed in nature as parasites of insects and plants, suggested that the organism might pass the flagellated stage of its life history in some blood-sucking insect.[2] The theory proved an extremely difficult one to substantiate, however. The extremely slow development of kala-azar and of other infections due to leishmanias, and the lack of susceptible animals, both contribute to the difficulty of carrying out transmission experiments. The fact that blood-sucking insects which have been allowed to feed upon patients with kala-azar or oriental sore are found on subsequent dissection to contain flagellates does not justify the conclusion that the flagellates are leishmanias, because the latter cannot be distinguished on morphological grounds from the flagellates occurring in many insects. Even when the insects used have been bred in the laboratory, and one may reasonably assume that they are free from flagellate infection, it is essential to be able to demonstrate in some way that the strain isolated from the insect after feeding is the same as that obtained from the patient. The injection into animals of herpetomonads derived from insects and plants[3] has not given results which are sufficiently striking or constant to be considered convincing evidence of the pathogenicity of the inocu-

[1] Rogers, L., *Lancet*, 1904, lxxxii, 215.

[2] Rogers, L., *Proc. Roy. Soc. London, Series B*, 1906, lxxvii, 284.

[3] Discussed in Part I of this report.[7]

lated organisms; and the interpretation of the findings in such instances is complicated by the presence of natural flagellate infections in the animals employed (*e.g.*, mice, rats, amphibians, and birds).

The immunological properties of the leishmanias and herpetomonads have been very little studied. The writer,[4] in 1924, showed that at least three species of leishmanias could be distinguished by serological tests. Kligler[5] has also found that *L. brasiliensis* and *L. infantum* are immunologically distinct from one another and from *L. tropica*, while different strains of *L. tropica* are serologically identical. Wagener and Koch[6] recently carried out comparative serological tests with cultures of four strains of *Leishmania* and one strain of *Herpetomonas ctenocephali*, the latter a flagellate of the dog flea, which has been regarded as a possible vector of canine leishmaniasis. The anti-leishmania sera of these authors, which were prepared with killed cultures, had no effect upon *H. ctenocephali* but affected equally the different species of leishmanias.

Investigation of the chemical changes which may take place in various carbohydrate-containing culture media has not so far been utilized for the differentiation of species of *Herpetomonas*. Kligler[5] attempted to distinguish leishmanias by this means but was unable to draw any definite conclusion from his study with a small series of strains.

As a first step in the application of immunological and biochemical methods to the differentiation of flagellate species, a number of strains of herpetomonads have been isolated from various sources and compared in serological and fermenting properties with one another and with the leishmanias. Twelve strains in all have been cultivated as described in Part I of this report.[7] Two additional flagellate strains, *Herpetomonas ctenocephali* and *Trypanosoma rotatorium*, furnished by courtesy of Dr. E. E. Tyzzer, were included in the study. The leishmania strains were the same as those I have employed previ-

[4] Noguchi, H., *Proc. Internat. Conf. Health Prob. Trop. America*, Kingston, Jamaica, British West Indies, July 22 to August 1, 1924, published in Boston, 1924, p. 455.

[5] Kligler, I. J., *Tr. Roy. Soc. Med.*, 1925, xix, 330.

[6] Wagener, E. H., and Koch, D. A., *Univ. Calif. Pub. Zool.*, 1926, xxviii, 365.

[7] Noguchi, H., and Tilden, E. B., *J. Exp. Med.*, 1926, xliv, 307.

ously for immunological investigation.[4] Comparison of the biological characteristics of these eighteen strains of flagellates brought to light a number of interesting facts.

Serological Differentiation of Herpetomonads and Leishmanias.

Rabbits were immunized against *Herpetomonas oncopelti*, *H. lygæorum*, *H. culicidarum*, *H. muscidarum*, *H. media*, *H. parva*, *Leishmania tropica*, *L. brasiliensis*, *L. infantum*, and *L. donovani* by intravenous injections of 1 to 2 cc. of rich living cultures at 4 day intervals on four successive occasions. The animals were bled 9 days after the last inoculation. The action of the specific immune sera thus prepared was tested upon the homologous and heterologous strains by agglutination and by complement fixation.

For the agglutination test, 0.05 cc. of immune serum was added to 1 cc. of a saline suspension of culture. A drop of the mixture was examined immediately with the dark-field microscope and again after 30 minutes and after 18 and 24 hours. Gross examination of the tubes was carried out as well. Control tests with normal rabbit serum were made in each instance. The presence in the suspension under test of rosettes or agglomerated masses did not interfere with the observations, since the changes brought about by specific agglutination are easily to be recognized. Under the dark-field microscope both flagellum and cytoplasm are seen to be profoundly affected by the specific immune serum. The organisms become sluggish or motionless, the bodies swollen and uneven in contour, the flagella twisted irregularly and adherent to whatever comes in contact with them, and finally the bodies are broken up. With a strong specific serum the gross findings are similarly striking. Within 30 minutes the turbid suspension becomes granular and begins to sediment and after 18 hours the supernatant fluid is clear, and there is a compact whitish sediment at the bottom of the tube.

For test of the specific complement-fixing power of the sera, the anticomplementary titer of a given antigen was first determined and half the anticomplementary dose employed. The immune serum was inactivated by heating at 55°C. for 30 minutes, and the amount used was 0.1 cc. in a total volume of 1 cc., 0.9 per cent saline being the diluent. The quantity of guinea pig serum, employed as complement, was 0.1 cc. of a 40 per cent dilution, and of anti-sheep amboceptor 2 hemolytic units. Incubation for fixation (30 minutes) and subsequent hemolysis (30 minutes) was carried out at 37°C.

The results of the agglutination and complement fixation tests are recorded in Tables I and II. The striking phenomena observed were as follows:

1. The strong reactions of the flagellates with their homologous sera. These were clear-cut and unmistakable.

TABLE I.

Agglutination Tests.

Source of flagellate.	Antisera.										
	Anti-*oncopelti* (strain from *O. fasciatus*).	Anti-*lygæorum* (strain from *L. kalmii* No. 2).	Anti-*culicidarum* (strain from *Anopheles quadrimaculatus*).	Anti-*muscidarum*.	Anti-*media*.	Anti-*parva*.	Anti-*tropica*.	Anti-*brasiliensis*.	Anti-*infantum*.	Anti-*donovani*.	Normal rabbit serum.
O. fasciatus	++++	+	−	−	−	−	−	−	−	−	−
Oncopeltus sp.?	++++	+	−	−	−	−	−	−	−	−	−
L. kalmii No. 1	++++	+	−	−	−	−	−	−	−	−	−
" " " 2	+	++++	−	−	−	−	−	−	−	−	−
A. quadrimaculatus	−	−	++++	−	−	−	−	−	−	−	−
C. pipiens	−	−	++++	−	−	−	−	−	−	−	−
M. domestica	−	−	−	++++	−	−	−	−	−	−	−
Calliphora sp.? No. 1	−	−	−	−	++++	−	−	−	−	−	−
" " " 2	−	−	−	−	−	++++	−	−	−	−	−
A. syriaca No. 46	+	++++	−	−	−	−	−	−	−	−	−
" " " 213	++++	++	−	−	−	−	−	−	−	−	−
" *nivea*	++++	+	−	−	−	−	−	−	−	−	−
Oriental sore.											
L. tropica	−	−	−	−	−	−	++++	+	+	−	−
Espundia.											
L. brasiliensis	−	−	−	−	−	−	−	++++	−	−	−
Kala-azar.											
L. infantum	−	−	−	−	−	−	+	−	++++	+	−
" *donovani*	−	−	−	−	−	−	−	−	++	++++	−
Ctenocephalus canis.											
H. ctenocephali	−	−	−	−	−	−	−	−	−	−	−
Rana pipiens.											
T. rotatorium	−	−	−	−	−	−	−	−	−	−	−

++++, rapid, complete precipitation, agglutination involving both cytoplasm and flagella. +, definite effect on flagella, which are twisted and adhere to one another.

TABLE II.

Complement Fixation Tests.

Source of flagellate.	Antisera.										
	Anti-*oncopelti* (strain from *O. fasciatus*).	Anti-*lygæorum* (strain from *L. kalmii* No. 2).	Anti-*culicidarum* (strain from *Anopheles quadrimaculatus*).	Anti-*muscidarum*.	Anti-*media*.	Anti-*parva*.	Anti-*tropica*.	Anti-*brasiliensis*.	Anti-*infantum*.	Anti-*donovani*.	Normal rabbit serum.
O. fasciatus	++++	++	−	−	−	−	−	−	−	−	−
Oncopeltus sp.?	++++	++	−	−	−	−	−	−	−	−	−
L. kalmii No. 1	++++	++	−	−	−	−	−	−	−	−	−
" " " 2	++	++++	−	−	−	−	−	−	−	−	−
A. quadrimaculatus	−	−	++++	−	−	−	−	−	−	−	−
C. pipiens	−	−	++++	−	−	−	−	−	−	−	−
M. domestica	−	−	−	++++	−	−	−	−	−	−	−
Calliphora sp.? No. 1	−	−	−	−	++++	−	−	−	−	−	−
" " " 2	−	−	−	−	−	++++	−	−	−	−	−
A. syriaca No. 46	++	++++	−	−	−	−	−	−	−	−	−
" " " 213	++++	++	−	−	−	−	−	−	−	−	−
" *nivea*	++++	++	−	−	−	−	−	−	−	−	−
Oriental sore.											
L. tropica	−	−	−	−	−	−	++++	−	+	−	−
Espundia.											
L. brasiliensis	−	−	−	−	−	−	−	++++	−	−	−
Kala-azar.											
L. infantum	−	−	−	−	−	−	+	−	++++	++	−
" *donovani*	−	−	−	−	−	−	−	−	++	++++	−
Ctenocephalus canis.											
H. ctenocephali	−	−	−	−	−	−	−	−	−	−	−
Rana pipiens.											
T. rotatorium	−	−	−	−	−	−	−	−	−	−	−

++++, complete inhibition of hemolysis. +++, 25 per cent hemolysis. ++, 50 per cent hemolysis. +, 75 per cent hemolysis. −, complete hemolysis.

2. The absolute indifference to anti-leishmania immune sera of the herpetomonad flagellates and of *T. rotatorium*, and *vice versa*, an indifference showing that no serological affinity exists between the leishmanias and the other flagellates studied.

3. The reciprocal reactions which took place among the different strains of herpetomonads derived from milkweeds and the insects feeding on the latex of these plants. For example, the anti-*oncopelti* serum had an equally strong effect upon cultures isolated from *Oncopeltus fasciatus*, *Lygæus kalmii* No. 1, the Peruvian species of *Oncopeltus*, *Asclepias syriaca* No. 213, and *Asclepias nivea;* while the anti-*lygæorum* serum had the same effect upon the culture derived from *Asclepias syriaca* No. 46 as upon the homologous strain. If these serological reactions may be regarded as indicating species specificity of herpetomonads, we may conclude that *Herpetomonas oncopelti* was present in three insects and two plants and *H. lygæorum* in one insect and one plant, or, viewing the matter from another point of view, that the insect, *Lygæus kalmii*, may harbor either *H. oncopelti* or *H. lygæorum*, as may also the plant, *Asclepias syriaca*.

4. The serological independence of three strains of herpetomonads derived from flies (a house fly and two bluebottle flies).

5. The serological identity of the two strains isolated from mosquitoes, one from *Anopheles* (adult) and the other from *Culex* (larva).

6. The inability of heterologous immune sera to affect *Herpetomonas ctenocephali* and *Trypanosoma rotatorium*.

The reactions among the leishmanias were similar to those previously reported, *L. tropica*, *L. brasiliensis*, and *L. donovani* being serologically distinct, and *L. infantum* related to *L. donovani*.

Fermentation of Carbohydrates by Herpetomonads and Leishmanias.

Leptospira medium, to which litmus had been added as an indicator of acid formation, and which contained the carbohydrate in a concentration of 1 per cent, was employed. All the flagellates grew luxuriantly on this medium at room temperature. The tubes were allowed to stand for 18 days before the final results were recorded, in order to permit the fermentation to reach the maximum point. Some of the sugars were split slowly. Table III shows the degree of acid formation, indicative of carbohydrate cleavage, observed in the various media.

TABLE III.

Fermentation Tests.

Results read after 18 days at 20°C.

Source of flagellate.	Carbohydrate in the medium.																
	Glucose.	Levulose.	Mannose.	Saccharose.	Raffinose.	Inulin.	Galactose.	Maltose.	Salicin.	Xylose.	Mannitol.	Dextrin.	Arabinose.	Amygdalin.	Lactose.	Dulcitol.	Rhamnose.
O. fasciatus	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	+	−	−	−	−
Oncopeltus sp.?	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	+	−	−	−	−
L. kalmii No. 1	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	+	−	−	−	−
" " " 2	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−	−	−	−
A. quadrimaculatus	++++	++++	++++	++++	++++	++++	+++	+++	++++	+	+++	+	−	++++	−	−	−
C. pipiens	++++	++++	++++	++++	++++	++++	+++	+++	++++	+	+++	+	−	++++	−	−	−
M. domestica	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	+	−	+	−	−
Calliphora sp.? No. 1	++++	++++	++++	++++	++	++++	+	−	−	−	−	−	−	−	−	−	−
" " " 2	++++	++++	++++	++++	++	−	++	−	−	−	−	−	−	−	−	−	−
A. syriaca No. 46	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−	−	−	−
" " " 213	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	+	−	−	−	−
" *nivea*	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	+	−	−	−	−
Oriental sore.																	
L. tropica	+++	+++	+++	+++	++	+?	−	−	−	−	−	−	−	−	−	−	−
Espundia.																	
L. brasiliensis	+++	+++	++	+++	++	−	−	−	−	−	−	−	−	−	−	−	−
Kala-azar.																	
L. infantum	+++	+++	+++	+++	+++	−	−	−	−	−	−	−	−	−	−	−	−
" *donovani*	+++	+++	+++	+++	+++	−	−	−	−	−	−	−	−	−	−	−	−
Ctenocephalus canis.																	
H. ctenocephali	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Rana pipiens.																	
T. rotatorium	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

++++, deep red, turbid (coagulation of serum); +++, deep red, serum clear; ++, markedly red, serum clear; +, pink, serum clear; −, no change in original bluish color.

The great variability in the fermentative faculty of the different flagellates is at once apparent. Some fermented practically all of the carbohydrates tested, others only a few, and still others none at all. *H. oncopelti* attacked 13 of the 17 carbohydrates tested, *H. lygæorum* only 3. *H. culicidarum* affected 13 carbohydrates, *H. muscidarum* 14, and each had a distinguishing feature, the former fermenting amygdalin but not lactose, the latter slightly attacking lactose but not affecting amygdalin. All the leishmania strains fermented glucose, levulose, mannose, saccharose, and raffinose, and *L. tropica* affected inulin slightly. *H. parva*, the strain from *Calliphora* No. 2, fermented the same sugars as the leishmanias, and galactose in addition. *H. media*, from *Calliphora* No. 1, also resembled the leishmanias but differed from them, and from *H. parva*, in fermenting inulin energetically. It affected galactose only slightly. Only dulcitol and rhamnose were unaffected by any of the flagellates, and of the 18 strains of flagellates tested, *H. ctenocephali* and *T. rotatorium* were the only ones which did not ferment any of the carbohydrates. *H. muscidarum* was the only flagellate to ferment lactose, and arabinose was affected only by *H. muscidarum* and *H. oncopelti*. Amygdalin was fermented only by *H. culicidarum*.

Whether the observed differences will persist during prolonged cultivation remains to be seen.

DISCUSSION.

The classification adopted for the species of *Herpetomonas* isolated in the course of the present investigation requires some comment, in view of the fact that heretofore the custom has been to regard the flagellates found in a given host as specific for that host and to name each strain in a way that indicates its source. If this custom had been rigidly followed, however, it would have been necessary to select several names for organisms which in cultural characteristics, in immunological properties, and in biochemical activity behave in the same way, as, for example, the strains isolated from *Oncopeltus fasciatus*, the Peruvian species of *Oncopeltus*, *Lygæus kalmii* No. 1, *Asclepias syriaca* No. 213, and *Asclepias nivea*. We already had evidence that the strain in *A. nivea* was the same as that in *O. fasciatus*, since the healthy *nivea* plants had been experimentally infected by the feeding

of specimens of *O. fasciatus* known by feces examination to harbor a *Herpetomonas*.[8] That this same species of flagellate should be present in the normal plant host of *O. fasciatus* (*Asclepias syriaca*) was to have been expected. Nor is it surprising that the same organism should be isolated from another insect which feeds on *A. syriaca*, namely, *Lygæus kalmii*. The infection of this insect could have taken place through ingestion either of infected plant latex or of infected dejecta of *O. fasciatus* deposited on the plant. The ingestion of feces by the bugs has been observed during the course of the experiments, and the insects also have been seen with proboscis inserted into the abdominal cavity of other bugs which were dead or moribund. All the facts, therefore, justify us in regarding these four strains as the same species. The isolation of the same flagellate from the Peruvian species of *Oncopeltus* is perhaps less readily explainable, though not remarkable.

Similarly the facts support the conclusion that the strains isolated from *Lygæus kalmii* No. 2 and *Asclepias syriaca* No. 46 constitute another species. While these two strains showed a slight group reaction toward the anti-*oncopelti* immune serum, they differed greatly from *H. oncopelti* in their action on carbohydrates, fermenting only three sugars instead of thirteen. They average slightly smaller in size, both under natural conditions and in culture, though the morphological differences alone would probably not enable one to discriminate the species. That *Lygæus kalmii* should harbor two types of *Herpetomonas* is not remarkable, and that its plant host should have become infected with both of them is wholly reasonable.

The striking morphological differences among some of the strains under discussion, as they occur in nature, appear to be due to the environment, since they disappear on cultivation under the same conditions. The typical herpetomonad of the plant latex has a flat, ribbon-like body, usually with one or two twists, the cytoplasm is clear, almost hyaline, and the flagellum short—about half the length of the body—and rather straight. The body of the herpetomonad

[8] These Haitian milkweeds, as already stated in Part I of this report, were presented by Dr. Francis O. Holmes, of the Boyce Thompson Institute for Plant Research, Yonkers, N. Y., who had infected them by allowing infected specimens of *Oncopeltus fasciatus* to feed upon the seed pods.

seen in the insect gut is usually cylindrical in appearance and is filled with volutin granules; the flagellum is as long as the body or longer, and rather flexible. The flagellates seen in the latex of *A. nivea*, though known to have come from *O. fasciatus*, are charactersitic of the plant type, and the same is true of *H. oncopelti* and *H. lygæorum* as they occur in *A. syriaca*.

In the case of the flagellates isolated from *Calliphora*, we were confronted with the alternative of identifying two strains, distinct morphologically, serologically, and in biochemical activity, with *H. calliphoræ*, or of choosing new names for them. The former course was manifestly unreasonable. The names *H. media* and *H. parva* were selected for these organisms because of the morphological differences between them, but there are differences as well in biological characters whereby the organisms may be identified at any time. We chose a new name also for the strain isolated from *Musca domestica*, because it appeared hardly legitimate to assign to *Herpetomonas muscæ domesticæ* the characteristics which this particular strain had shown.

The flagellates isolated from mosquitoes came from two different host genera, *Anopheles* and *Culex*, but could not be differentiated morphologically, culturally, or in biochemical properties. The name selected for the organism represented by these two strains was *Herpetomonas culicidarum*, which indicates its occurrence in two genera of the family Culicidæ and denotes also the specific properties by which it may be differentiated from the other flagellates of the series, and perhaps also from other flagellates of mosquitoes.

SUMMARY.

Serological reactions and fermentation tests have been employed in the present investigation as a means of differentiating various strains of herpetomonads from one another as well as from leishmanias. The twelve strains of herpetomonads isolated from insects and plants all proved to be serologically unrelated to any of the leishmanias, and were distinguishable from them by the manner in which they affected various carbohydrates.

Three of the strains of herpetomonads tested had been isolated from milkweeds (*Asclepias syriaca* and *A. nivea*) and four from bugs which feed on the latices of these plants (*Oncopeltus fasciatus*, *Oncopeltus sp.?* from Peru, and *Lygæus kalmii*). When tested for their serological

and carbohydrate-fermenting properties, however, the seven strains proved to be of two kinds only, one represented by the strain first isolated from *Oncopeltus fasciatus* and hence named *H. oncopelti*, the other by *H. lygæorum*, so named because it was first isolated from *Lygæus kalmii*. Serologically there was a certain degree of group reaction among the flagellates of these two types, but in their action upon carbohydrates they were entirely different, *H. oncopelti* splitting thirteen carbohydrates, *H. lygæorum* only three.

Three strains of herpetomonads isolated from flies proved to be distinct both in serological properties and in their action upon carbohydrates. One, derived from the house fly, and called *H. muscidarum*, was able to ferment most of the carbohydrates tested, including lactose which was not affected by any of the other strains. The other two, isolated from bluebottle flies, behaved much the same as the leishmanias with regard to carbohydrate fermentation, attacking five of the same sugars. One of them fermented galactose in addition, the other both galactose and inulin.

Two strains from mosquitoes (*Anopheles* and *Culex*) behaved identically in serological reactions and also in fermentation tests. They are regarded as one species and have been named *H. culicidarum*. This organism ferments thirteen sugars, including amygdalin which no other organism of the series attacks.

One of the most striking phenomena observed was the entire lack of fermentative faculty on the part of *Herpetomonas ctenocephali* and *Trypanosoma rotatorium*. Neither of these organisms was affected by any of the immune sera prepared with other flagellates.

The serological specificity of *Leishmania tropica*, *L. brasiliensis*, and *L. donovani*, and the close relation between *L. donovani* and *L. infantum* were confirmed in the present study. These organisms could not, however, be differentiated by fermentation tests.

The data presented suggest that the biological characteristics of flagellates of the *Herpetomonas* group may be utilized with advantage for identification of a species which occurs in different environments and for separation of different species when they are found in the same environment. If the leishmania parasites pass the flagellated or herpetomonad stage of their life history in some invertebrate host, it may be possible by tests of the sort described to distinguish them from the non-pathogenic herpetomonads which are so widely distributed among insects and plants.

STUDIES ON X-RAY EFFECTS.

XV. The Prevention of Pigment Formation in the Hair Follicles of Colored Mice with High Voltage X-Ray.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plate 16.

(Received for publication, June 1, 1926.)

In the course of a study of x-ray dosage it was noted incidentally that the hair of rabbits became white within an area exposed to x-ray produced at high voltage (1). Since color and not the production of hair was affected it was considered desirable to gather more complete data in the hope that it might eventually throw some light on the biological action of the different wave-lengths of x-ray. The results of some preliminary experiments are recorded below.

Material.—In order that any change in color might be most easily detected black haired mice were chiefly used. The hair of such animals is evenly and deeply pigmented.

Methods.—The abdomens of the mice were shaved. The animals were then immobilized on their backs upon boards by binding them on with adhesive tape. Two holes about ⅜ inch in diameter and about ½ inch apart had been punched in a shield of rubber and lead, and the rubber cement from adhesive tape applied to a small area about the holes by sticking a piece of tape of proper size over the holes and saturating the cloth with xylol. The cloth could then be peeled off leaving its adhesive adhering to the rubber and lead shield. This perforated shield was now adjusted over the shaved abdomen of the mouse where it adhered because of the adhesive. The edges of the shield were fastened to the board by glass thumb tacks. In exposing the shielded animals to the x-ray both holes were left uncovered for the period desired and then the anterior hole was shielded while the posterior opening was rayed for 10 minutes longer. Thus two exposures of different lengths were made on each animal. Two mice were used for each period of exposure employed.

The X-Ray Outfit.—The x-ray machine used was the one designed by Clark (2),

and was operated at 70 kilovolts and 8 milliamperes. Exposures ranged from 20 to 100 minutes at 36 mm. target distance.

Observations.—The most complete color change was obtained in animals that had been exposed for 50 to 100 minutes. The hair in the shaved but unexposed areas began to reappear in about 2 weeks while that in the exposed regions was not visible until 1 week later. When the latter did come in it was entirely white; and after the lapse of 1 month in all it was as long and thick as the rest of the hair (Fig. 1). The hair in the areas exposed from 70 to 100 minutes grew more rapidly than it did in the spots receiving 50 to 60 minute doses. At the end of 3 months the regions rayed for 50 to 60 minutes were rather sparsely covered with hair while the hair in the spots receiving longer exposures grew back in full vigor. The hair in areas exposed for 20 to 50 minutes did not suffer as complete a color change as when the raying had been for longer periods. Only a few scattered hairs in these areas became whitened, the great mass reappearing in the natural color.

$2\frac{1}{2}$ months after one of the animals had received 90 and 100 minute exposures upon the abdomen it was noted that a white spot was appearing on its back in a position directly opposite the spot that had received the 90 minute dose (Fig. 2). Under the binocular this white spot, which was slightly larger than the corresponding one on the abdomen, was seen to be made up of a mixture of white and black hairs. The white hairs were not banded, with the outer portion the original black, as might be expected had the hair continued to grow after the hair follicle had lost its power of producing pigment. A second area on the back corresponding to the spot on the abdomen exposed for 100 minutes appeared at first as a white line which after about 10 days extended to form a complete circle. Evidently the rest of the area of exposure had been shielded by the pelvic bones or some other internal obstruction to the rays. Almost 2 weeks after these dorsal white spots appeared on mice rayed for 90 and 100 minutes similar areas appeared on the mice exposed for 70 and 80 minutes. At the time of writing, 3 months after the beginning of the experiment, none of the animals rayed for shorter intervals have developed white spots on the back.

After the hair had grown again to its original length in the exposed areas one of the white spots was shaved. When the hair came in once more it was as white as before.

DISCUSSION.

Comparatively little seems to be known about the pigment production in hair. Pigment cells of ameboid shape are found clustered about the papillæ at the base of the follicle and pigment granules have been described within and between the cells of the cortex of the hair of human beings. In addition to the "solid" colors that result from the presence of these granules, pigments in solution are said to stain the cortical cells. Hair is said to be pure white only when pigment is lacking and air is present between the cells of the medulla and the cortex. In cases where air is missing the hair appears grey, never white (3).

Whatever the actual processes responsible for the presence of pigment in hair it is evident that in some way hard x-rays interfere with it permanently. In addition to destroying the color-developing power of the hair, the structure of the cortical cells must be altered in order to permit the entrance of air between them, since otherwise, according to the authority above cited, the hair would not be pure white.

As is evident from the symmetry of the white areas, the effect of the x-rays is due to the direct impingement of them and the injury does not spread beyond the open areas of the shield. Where the effect is seen on the back the force of the rays has been modified by the obstructions to them existing between the ventral and dorsal surfaces. Shorter exposures took longer postexperiment intervals to produce any effects on the pigment in the dorsal hair than did the longer doses.

It is of interest to note the great length of time, $2\frac{1}{2}$ months, between the exposure and the appearance of white hairs on the back of the mouse. There was no dropping out in mass of this hair but the colored hair was gradually replaced with white. This may mean that the changes induced are cumulative. No gradation from black to white was encountered with the ultimate result of an abrupt cessation of pigment production. As far as could be determined none of the hairs were banded, *i.e.* black at the tip and white at the base, but were entirely white.

A similar loss of pigment has recently been reported by Coolidge (4) following exposures to the cathode rays. In his cases the hair appeared to grow more rapidly than in the surrounding unexposed area. In the earlier experiments referred to above (1) it was not unusual to find that the hair in an exposed area grew out as a tuft before that in the surrounding shaved area had reappeared. No such increased rate of growth was observed during the present work. Indeed after some of the shorter exposures the growth of the hair was definitely retarded.

SUMMARY.

1. Hard x-rays prevent the formation of pigment in the hair follicles of mice, with result that hair previously black comes in white.

2. Exposure of the ventral side of a mouse to the x-rays causes the hair on the back to come in white about 40 to 50 days after it has whitened the hairs on the abdomen.

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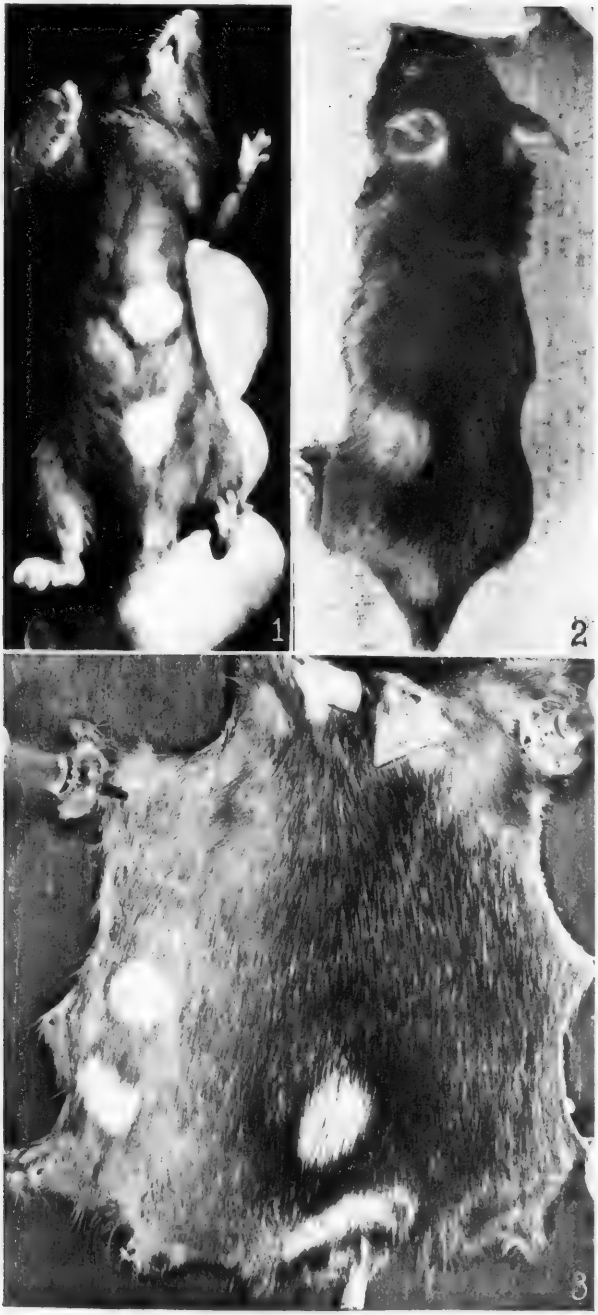
EXPLANATION OF PLATE 16.

FIG. 1. Ventral view of black haired mouse showing the two circular patches of white hair which appeared after exposure of these areas to high voltage x-rays for 90 and 100 minutes.

FIG. 2. Dorsal view of the same mouse showing two areas on the back corresponding to those on the lower side. The dorsal spot is not as well marked for reasons discussed in the text. The whitened hairs appeared about 1 month later than those shown in Fig. 1.

FIG. 3. The skin of the mouse pictured in Figs. 1 and 2 stretched to show in one photograph both the ventral and dorsal spots.



(Hance and Murphy: Studies on x-ray effects. XV.)

UNIV. OF MICH.

BIOLOGY OF BACTERIUM LEPISEPTICUM.

III. Physical, Cultural, and Growth Characteristics of Diffuse and Mucoid Types and Their Variants.

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(Received for publication, May 10, 1926.)

We have been engaged in the study of *Bact. lepisepticum* infection in rabbits with the purpose of finding out the mechanism which governs its natural prevalence. Thus far we have determined three factors which play essential parts: (1) host susceptibility; (2) microbic distribution; and (3) microbic virulence. The first two of these have been discussed in previous papers (1); in this and the following report we shall present the results of our investigations of the infecting agent, *Bact. lepisepticum*.

Occurrence.

During the past 4 years we have obtained, from rabbits at the Rockefeller Institute animal house, two distinct types of *Bact. lepisepticum*—so called "D" forms and mucoid forms. De Kruif had previously, in 1920 to 1922, obtained strains of typical rabbit septicemia bacteria from the Rockefeller Institute stock which he observed to dissociate *in vitro* from the original so called "D" type to a "G" type (2); and he later recovered these "G" variants directly from the nasal passages of clinically normal rabbits (3). In 1922 and 1923, we isolated similar "D" and "G" strains from the nasal passages of apparently healthy rabbits, and from rabbits suffering from spontaneous pneumonia, snuffles, and otitis media; but since that time, only mucoid strains and their variants have been recovered. A large percentage of the Rockefeller Institute rabbits has continued to harbor *Bact. lepisepticum*, but always in the mucoid form. Several hundred cultures, taken from rabbits at a farm in New City, N. Y., over a period of 12 months, were mucoid; and *Bact. lepisepticum* of the mucoid type proved to be

the incitant of an epidemic of snuffles and pneumonia in a rabbit stock at Ray Brook, N. Y.[1] We conclude, therefore, that mucoid strains of *Bact. lepisepticum* have supplanted the "D" strains in the rabbits at the Rockefeller Institute, and are, at present, the prevailing type in at least two other rabbit communities.

Morphology and Staining Characteristics; Colony Formation; Metabolic Activities.

All types of *Bact. lepisepticum* have the general characteristics of the Pasteurella group. They are small, blunt, Gram-negative, non-motile rods, with deep staining polar bodies. Varying amounts of acid, without gas, are formed within 24 to 48 hours by cultures in peptone broth, containing 1 per cent dextrose, levulose, xylose, saccharose, and mannitol. About 50 per cent of all strains ferment glycerol after 4 to 5 days of incubation. Maltose, lactose, salicin, dulcitol, inulin, arabinose, and rhamnose are not fermented.

Nitrates are reduced to nitrites; the indole test (Ehrlich) is positive. Litmus milk is not acidified.

"D" strains differ somewhat in morphology and staining characteristics from "G" variants and also from mucoid forms and their variants. The special characteristics of each type are found by examining fresh films of living bacteria with dark-field illumination, or by staining them with Wright's modification of Romanowsky's blood stain. Under these conditions, Type "D" cells appear to consist of a rod-shaped, refractile, pink-staining substance, containing two dark, purple-staining polar bodies. They have a slow Brownian movement. Type "G" variants are much smaller: the two polar bodies seem to touch each other, and the refractile, pink-staining, "cytoplasmic" substance is scarcely visible. Brownian movement is rapid. The mucoid forms are twice as large as the "D" types. Their polar bodies are widely separated by the refractile "cytoplasmic" substance which seems to overflow into an outer band or capsule.[2] Brownian oscillations are slow.

[1] This spontaneous epidemic has been studied by Dr. D. T. Smith, and will be described later in detail.

[2] This material was not stained with Hiss or Muir stains, nor did Wright's fluid color the "capsules" of pneumococci. Opinions differ as to the presence or absence of "capsules" on bacteria of the Pasteurella group (4).

Besides these specific differences in morphology, other variations in size occur, which are associated with the age of the culture (5).[3] During the period of logarithmic growth of a broth or agar culture of "D," mucoid, or variant strains, individual cells appear very large. This is due, apparently, to an increase in amount, or swelling of the pink-staining, "cytoplasmic" substance. The polar bodies are enlarged also, and stain a reddish purple. Chains, filamentous forms, and other irregularities are numerous. After 24 hours incubation, when the number of organisms is constant, at about 1 billion per cc., the cells become uniform and less than one-half their former size. And finally, after incubation at 37°C. for more than 3 or 4 days, most of the bacteria are still smaller and stain pale blue.

The "D" and "G" colonies have been described by De Kruif as follows: The "D" colonies "were whitish, with rather opaque, glistening centers, fading into translucent outer zones. Their borders were regular. They exhibited marked fluorescence, both by daylight and by artificial light. The surface colonies of Type "G" were somewhat smaller, translucent, and bluish in color, had irregular, serrated edges, and showed little or no fluorescence" (2, 3). In addition to these two colony forms as described, we found, in old broth cultures of "D" strains, a white, opaque colony, slightly fluorescent. From this intermediate colony, a mixture of "D" and "G" types of bacilli was invariably obtained.

Davis (6) has described the colony form of hemorrhagic septicemia bacteria recovered from spontaneous abscesses of rabbits as being moist, viscid, and slightly spreading. The mucoid strains which we have encountered are probably similar. The colonies are large and fluorescent, with whitish, opaque centers and translucent, flowing mucoid margins. The "G" variants of these mucoid strains have a characteristic pin-head colony, convex, bluish, with regular edges, firm in texture, somewhat adherent to the agar, and difficult to emulsify.

Peroxidase is present in less amounts in the "D" and mucoid types than in the "G" variants (7). Peroxide formation could not be demonstrated by direct methods (8); there was, however, greenish

[3] Clark has described the same phenomena in other bacterial species (5).

discoloration beneath the surface of blood agar tube cultures of "D," mucoid, and "G" types, which, according to McLeod, is indirect evidence of the presence of peroxide (9).

TABLE I.

Agglutination of "D," Intermediate, and "G" Cultures of Bact. lepisepticum in Lactate-Lactic Acid Buffer Solution.

Rabbit No. and culture No.	Colony type.	pH range of buffers.									
		4.7	4.5	4.1	3.8	3.5	3.3	3.0	2.7	2.4	Control.
74	D	–	–	–	–	C.	C.	C.	–	–	–
90	"	–	–	–	–	C.	C.	C.	–	–	–
Rivers.	"	–	–	–	–	C.	C.	C.	–	–	–
157	"	–	–	–	–	C.	C.	C.	–	–	–
36	"	–	–	–	–	C.	C.	C.	–	–	–
24	"	–	–	–	C.	C.	++	–	–	–	–
115	"	–	–	–	C.	C.	C.	–	–	–	–
137	"	–	–	–	C.	C.	C.	C.	–	–	–
79	I	–	–	C.	C.	C.	–	–	–	–	–
88	"	–	–	C.	C.	C.	–	–	–	–	–
3 W	"	–	–	C.	C.	C.	–	–	–	–	–
203	"	–	C.	C.	C.	C.	C.	C.	–	–	–
125	"	–	C.	C.	C.	C.	C.	–	–	–	–
179	"	–	C.	C.	C.	C.	C.	C.	–	–	–
186	G	+	C.	C.	C.	+	–	–	–	–	–
201	"	C.	C.	C.	C.	–	–	–	–	–	–
119	"	C.	C.	C.	C.	–	–	–	–	–	–
Rivers.	"	C.	C.	C.	–	–	–	–	–	–	–
188	"	C.	C.	C.	C.	–	–	–	–	–	–
135	"	C.	C.	C.	C.	C.	+	–	–	–	–
35	"	C.	C.	C.	–	–	–	–	–	–	–

– No agglutination.
+ Slight agglutination.
++ Good agglutination; supernatant turbid.
C. Complete agglutination.

Physical Characteristics.

Acid agglutination titrations were made on a large number of freshly isolated cultures of *Bact. lepisepticum*, to compare the isoelectric points of "D" and mucoid types.

De Kruif found that the acid agglutination zone of his "D" types was pH 3.0 to 3.5, and of "G" variants, pH 3.5 to 4.7 (10). He noted also that the agglutination zone was affected by the buffer solutions employed.

We employed the standard lactate-lactic acid buffers and technique used by Northrop and De Kruif (11). 16 to 18 hour broth cultures were washed and centrifugalized four times with distilled water. Equal parts of cell suspension and buffer were placed in the water bath at 55° and readings were made at 6 and 18 hours.

TABLE II.

Acid Agglutination of Mucoid Strains.

Control No.	Colony type.	pH									
		4.7	4.5	4.1	3.8	3.5	3.3	3.0	2.7	2.4	Control.
Rivers.	D	–	–	–	C.	C.	C.	C.	–	–	–
"	G	–	++	C.	C.	C.	++	–	–	–	–
Brown's	"	C.	C.	C.	C.	C.	–	–	–	–	–
"	"	C.	C.	C.	C.	C.	–	–	–	–	–
"	I (intermediate).	–	++	C.	C.	C.	C.	C.	++	–	–
"	M (mucoid).	–	–	–	++	C.	C.	C.	C.	–	–
DC 30	"	–	–	–	C.	C.	C.	C.	C.	–	–
653	"	–	–	–	C.	C.	C.	C.	++	–	–
544	"	–	–	–	C.	C.	C.	C.	C.	+	–

– No agglutination.
+ Slight agglutination.
++ Good agglutination; supernatant turbid.
C. Complete agglutination.

Table I shows the result of one such test on twenty-one freshly isolated "D," "intermediate," and "G" variant strains. The eight "D" strains flocculated completely between pH 3.0 and pH 3.5; the "G" strains between pH 3.5 and pH 4.7; and six cultures from "intermediate" colonies showed a wide range, characteristic of mixtures of the "D" and "G" types (12).

The acid agglutination zone of mucoid types proved similar to that of the "D" strains. For example, Table II shows the results of a

test in which four mucoid cultures were compared with a "D" type, three "G," and one "intermediate" strains. The "D" culture flocculated between pH 3.0 and pH 3.8, the mucoid strains from pH 2.7 to pH 3.8, the "intermediate" from pH 2.7 to pH 4.5, and the "G" from pH 3.5 to pH 4.7.

Type differences in stability of suspension were marked. Mucoid strains did not flocculate completely after 6 to 8 hours centrifugalization at high speed. "D" strains, however, came down in 3 to 4 hours, and "G" strains in ½ to 1 hour.

TABLE III.

Rate of Migration of Bact. lepisepticum, Types "D," "G," and Mucoid in Glycine-Acetate-Phosphate Buffer, pH 7.4.

Type strain.	No. of seconds to travel 83.33μ.	Potential $\left(\frac{83.33}{\frac{\text{No. sec.}}{5}} \times 14\right)$.
	sec.	
Rivers "D."	6.3	−37
" "G."	8.9	−26.2
M 555	6.1	−38.2
M 518	5.8	−40.2
Rivers "D"	5.7	−40.9
M 587	6.0	−39.0
M 183	5.7	−40.9

Potential determinations were made with the Northrop type of cataphoresis cell (13). Cell suspensions were washed thoroughly and suspended in glycocoll buffer at pH 7.4, as described by Northrop and De Kruif (11).

The results of these observations are recorded in Table III. Rates of migration of the "D" type and "G" variant were found to be similar to those reported by Northrop and De Kruif; those for the mucoid strains proved similar to the "D" and were considerably higher than the "G" variants.

Growth Requirements and Characteristics.

The different types of *Bact. lepisepticum* fail to grow in buffered asparagine and tryptophane media at atmospheric or reduced oxygen

tension, and with or without hemoglobin, iron salts, or other oxygen-absorbing substances. But a 1 per cent peptone solution, or meat extract or infusion broth is adequate for abundant growth of all types, when inoculations of sufficient size are made. In media of the latter sort, specific differences in the manner of growth of "D," "G" variant, and mucoid forms are apparent. The behavior of the "D" and "G" variant strains has been described before, but for the sake of comparison, the main facts may be summarized again as follows:

De Kruif noted that "D" strains, inoculated into broth or peptone solutions, gave rise to "G" variant forms after 48 to 72 hours incubation (2). Later, we made a more detailed study of the growth of this type (14, 15), and noted the following facts: (1) that "D" strains, inoculated into aerobic meat infusion broth, pH 7.4, in quantities less than 100,000 per cc., did not live; (2) that larger seedings multiplied after a period of lag, until a maximum number of 1,000,000,000 per cc. was reached; (3) that after 48 to 72 hours incubation, "G" variants appeared in increasing numbers, until they replaced entirely the original "D" forms. Furthermore, we noted that (4), if the oxygen tension of the broth was lowered mechanically or by the addition of hemoglobin or other iron-containing compounds, inoculations as small as two or three bacilli per cc. invariably multiplied logarithmically, with no appreciable lag until, after 9 to 10 hours, a maximum number of 1,000,000,000 per cc. was reached. Under these latter conditions, the appearance of Type "G" variants was inhibited indefinitely. Finally, we noted that "G" variant strains grew well in broth at either atmospheric or reduced oxygen pressure.

The mucoid strains have now been studied similarly, with the following results.

The first test was planned to determine the smallest number of mucoid organisms which would multiply in tubes containing 5 cc. of plain broth, or plain broth plus hemoglobin.

Test 1.—Three mucoid cultures from a rabbit farm at New City, N. Y., and one from the Rockefeller Institute stock were employed. DC 30 and DH 5 were obtained from the nasal passages of animals with snuffles; DC 12 from a lung abscess, and 544 from a healthy nasal carrier. 24 hour plain broth cultures were seeded in ½ cc. amounts, in dilutions varying exponentially from 1 to 9, into tubes contain-

ing 5 cc. of plain broth and into others containing the broth plus ½ cc. of a 1/100 solution of hemolyzed rabbit red blood cells. The tubes were then incubated and examined at frequent intervals for evidence of growth.

The results are shown in Table IV. All hemoglobin broth cultures showed maximum growth within 24 hours, indicating that an inoculation of less than ten organisms was sufficient for multiplication. The plain broth cultures of DC 30, DC 12, and DH 5, seeded with 300,000 or more, and No. 544, with 3000 or more, showed good growth at 24 hours. Other plain broth cultures, with an original inoculation of

TABLE IV.

Growth of Mucoid Strains in Plain Broth and Broth Plus Hemoglobin.

Culture No.	Broth medium.	No. per cc. in millions in original cultures.	Dilution.							
			10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
DC 30	Plain.	360	++	++	48 hrs.	90 hrs.	90 hrs.	90 hrs.	90 hrs.	0
"	Hemoglobin.	360	++	++	++	++	++	++	++	++
DC 12	Plain.	330	++	++	48 hrs.	48 hrs.	48 hrs.	48 hrs.	48 hrs.	
"	Hemoglobin.	330	++	++	++	++	++	++	++	++
DH 5	Plain.	360	++	++	90 hrs.	90 hrs.	90 hrs.	0	0	0
"	Hemoglobin.	360	++	++	++	++	++	++	++	++
544	Plain.	370	++	++	++	++	90 hrs.	90 hrs.	0	0
"	Hemoglobin.	370	++	++	++	++	++	++	++	++

++ = Maximum growth in 24 hours.

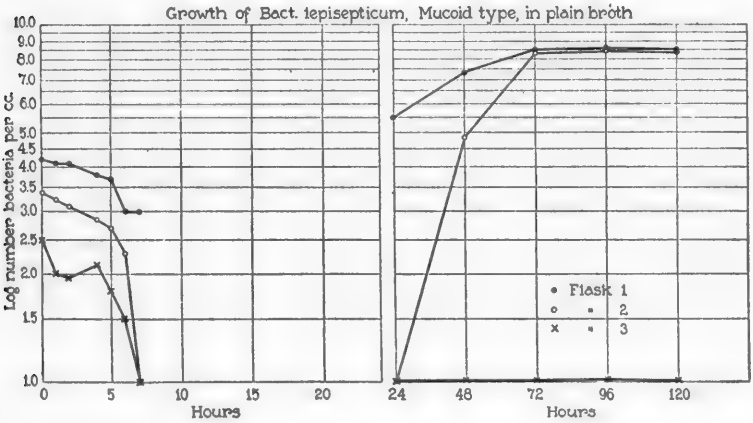
less than ten organisms, grew after a lag of from 48 to 90 hours. 9 days later, "G" variants were found in the plain broth cultures of DC 12; the other cultures remained mucoid throughout the 2 weeks period of observation.

This lag in the growth of mucoid strains in plain broth was investigated further by the serial counting method.

Test 2.—A 24 hour plain broth culture of DC 30 was inoculated, in varying amounts, into three 500 cc. Erlenmeyer flasks, each containing 150 cc. of plain broth. Flask 1 received 1 cc. of a 1:100 dilution; Flask 2, 1 cc. of a 1:1000; and Flask 3, 1 cc. of a 1:10,000 dilution. The number of bacilli per cc. in the flasks was

determined at once by the plating method; the flasks were then incubated and counts were made at frequent intervals.

The growth curves are plotted in Text-fig. 1. The original number of 15,000 per cc. in Flask 1 dropped, after 7 hours, to 1000; rose at 24 hours to 300,000; and reached a maximum of 300,000,000 per cc. within 72 hours. In Flask 2 the original count of 2460 dropped to zero within 7 hours. The next day, the count was still zero, but on the 3rd day the maximum number of 350,000,000 per cc. was reached. The number of bacteria in Flask 3 dropped within 7 hours from 300 to zero, and no subsequent growth occurred during the 120 hour period of observation.



TEXT-FIG. 1. Growth of *Bact. lepisepticum*, mucoid type, in plain broth.

The behavior of the mucoid strain in blood broth was then tested in the same manner.

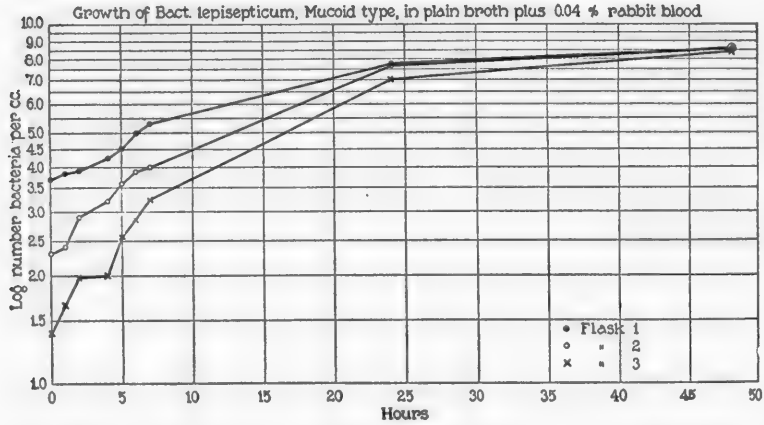
Test 3.—The same strain, DC 30, was grown for 18 hours in a tube of plain broth and then seeded, in 1 cc. amounts, at dilutions of 1:100, 1:1000, and 1:10,000, into three flasks of 0.04 per cent hemolyzed rabbit blood in 150 cc. of broth. The flasks were incubated and the number of organisms per cc. was determined at frequent intervals by the serial counting method.

The growth curves are shown in Text-fig. 2. The numbers of bacteria inoculated into each flask—4800 in Flask 1; 200 in Flask 2;

and 24 in Flask 3—increased immediately and at a more or less regular rate for 48 hours, when the maximum counts of 360, 450, and 282 millions per cc. were obtained.

One further experiment was planned to test the ability of sodium pentacyano-aquo-ferroate to act as a substitute for hemoglobin in broth media (13).

Test 4.—Two mucoid strains, DC 30 and DC 12, were grown 17 hours in plain broth and inoculated into 5 cc. tubes of plain broth and plain broth plus 2 per cent sodium pentacyano-aquo-ferroate, in dilutions increasing exponentially from 1 to 9. The two series of tubes were then incubated and examined subsequently for evidence of growth.



TEXT-FIG. 2. Growth of *Bact. lepisepticum*, mucoid type, in plain broth plus 0.04 per cent rabbit blood.

Table V shows the result. The mucoid strains grew as readily in the aquo salt broth as in blood broth, while in the plain broth tubes a characteristic lag occurred.

These experiments have emphasized several striking characteristics of the growth of mucoid strains of *Bact. lepisepticum*. First, a few hundred organisms, or more, inoculated into meat extract broth, survive and, after a lag continuing sometimes for more than 24 hours, multiply at a relatively slow rate, until a maximum number of 300 million to 400 million per cc. is reached. Second, "G" variants of the

mucoid strains appear slowly, or not at all, under these conditions. Third, hemoglobin or oxygen-absorbing substances added to meat extract broth supply conditions necessary for immediate multiplication after inoculation of less than ten organisms per cc. for 24 to 48 hours, until a maximum number of 300,000,000 to 400,000,000 is reached.

The next experiments were carried out to determine the behavior of "D" and mucoid cultures over long periods of time.

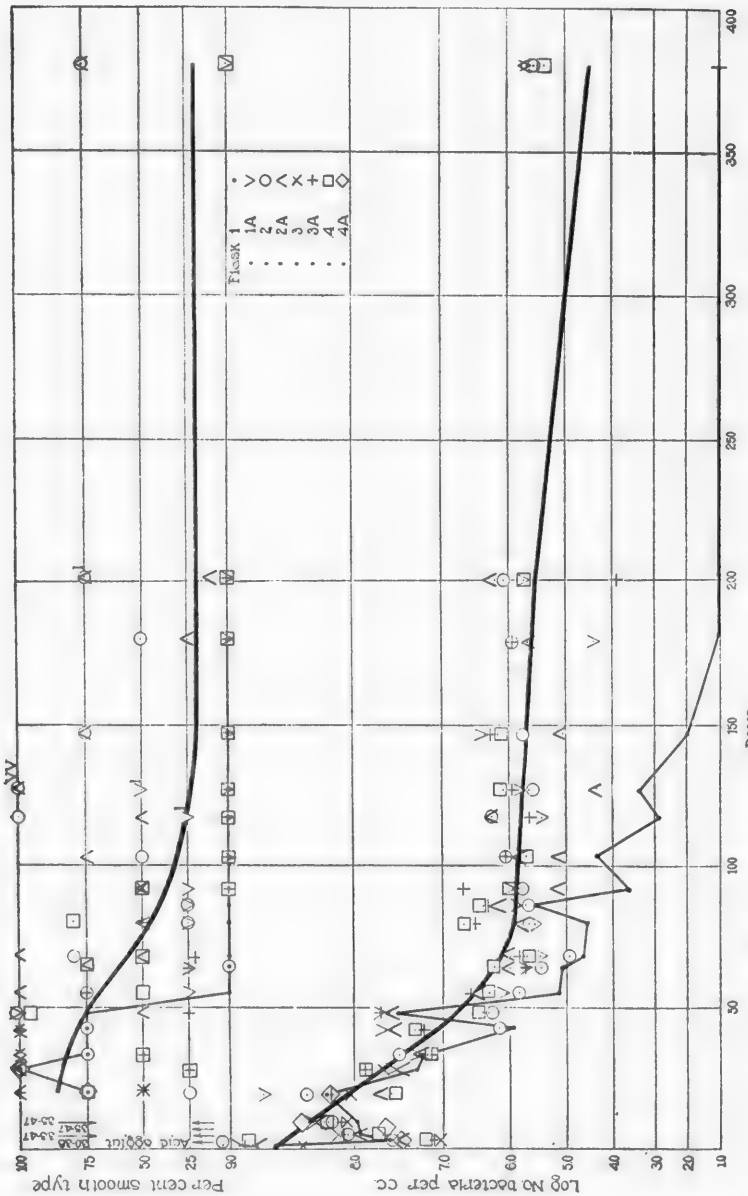
TABLE V.

Growth of Mucoid Strains in Plain Broth and Broth Plus Sodium Pentacyano-Aquo-Ferroate.

Culture No.	Medium.	Dilution.									Dissociation.
		10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{-0}	
DC 30	Plain broth.	++	++	++	4 days.	4 days.					0
"	Plain broth + aquo salt.	++	++	++	++	++	++	++	++		0
DC 12	Plain broth.	++	++	++	+	4 days.	4 days.	4 days.	4 days.	4 days.	+ 7 days.
"	Plain broth + aquo salt.	++	++	++	++	++	++	++	++	++	0

Test 5.—A 15 hour broth culture of the Rivers "D" strain was inoculated in ½ cc. amounts (about 500,000,000 bacteria) into each of eight Erlenmeyer flasks, containing 700 cc. of broth, plus differing amounts of rabbit red blood cells. Flasks 1 and 1 A contained 0.001 as many rabbit red blood cells per cc. as are in 1 cc. whole blood; Flasks 2 and 2 A contained 0.0001 of this quantity; Flasks 3 and 3 A, 0.00001 as much; and Flasks 4 and 4 A no blood. These cultures were then incubated for 12 months. From time to time, counts were made, acid agglutination titrations were carried out, type dissociation percentages were estimated, and atypical colonies and other phenomena studied.

The results are summarized in Text-fig. 3, and may be stated approximately as follows: 2 days after inoculation, the acid agglutina-



TEXT-FIG. 3. Survival of *Bact. lepisepticum*, "D" type, in plain and blood broth.

tion range of each culture was still in the "D" zone; on the 3rd and subsequent days it had widened to include both the "D" and "G" zones. This change was interpreted as indicating the appearance of "G" variants in the cultures. Further estimates made by plating methods showed a gradual decrease in the numbers of "D" colonies, and an increase in the "G" colonies. On the 200th day two of the remaining six flasks still contained "D" colonies. This and the slow rate of bacterial dissociation in plain broth are to be explained by the relatively large volume of broth and low oxygen tension in the flask used in this experiment. "Intermediate" colonies were noted in large numbers after the 100th day.

On the 34th day Flasks 3 and 4 A became contaminated and were discarded. The bacterial counts in five of the six remaining flasks were in close agreement, decreasing regularly from about 200,000,000 per cc. on the 5th day, to 3,000,000 on the 70th day. On the 200th and 377th days the counts averaged about the same. The count in Flask 1, however, dropped steadily after the 50th day and reached zero on the 180th day. This discrepancy cannot be explained at present.

Under the conditions of this experiment, therefore, the "D" type of *Bact. lepisepticum* survived for many months. During this period, the number of viable organisms per cc. dropped from its maximum to a point of approximately constant level, and at the same time, "intermediate" and "G" variants replaced the original "D" forms.

The curve of survival of mucoid cultures over long periods of time was similar to that of the "D" cultures; "G" variants appeared more slowly, however.

Serological Studies.

The antigenic properties of "D" and mucoid types were compared by means of direct and cross-agglutination tests.

Sera were prepared as follows: 17 hour blood broth cultures, heat-killed and living, washed once in saline, were injected intravenously or subcutaneously into rabbits which had not been previously exposed to *Bact. lepisepticum*. The animals were bled when the titre of the serum reached a maximum.

Agglutination tests were made with bacterial suspensions, washed four times in distilled water. The homologous strains flocculated so poorly, even when the pH

of the cell and serum mixtures was reduced to pH 6.5, that it became necessary to employ the "G" variant of each strain for the actual titrations. De Kruif found that "G" variants were antigenically similar to their homologous "D" strains (2), and we have observed this relationship between mucoid strains and their "G" variants. The variant "G" strains flocculated completely in serum dilutions of 10^3 or more, whereas the parent "D" or mucoid strains agglutinated with difficulty in serum dilutions as low as 10^2.

TABLE VI.

Direct and Cross-Agglutination of "G" Variants of "D" and Mucoid Strains of Bact. lepisepticum.

Antisera.	Culture.							
	Rivers "G"	No. 103 "G"	No. 329 Mucoid "G"	No. 544 Mucoid "G"	No. DC 12 Mucoid "G"	No. DC 30 Mucoid "G"	Snuffles (1) Mucoid "G"	Snuffles (4) Mucoid "G"
Rivers "D".....	3840*	960	0	0	0	0	0	0
No. 103 "D".....	960	960	0	60	0	0	0	0
" 329 mucoid.....	0	0	120	1920	120	480	960	480
" 544 "	0	0	0	480	960	240	480	960
" DC 12 mucoid.	0	240	470	3840	960	240	960	3840
" DC 30 "	0	0	1920	480	960	3840	60	—
Snuffles (1).....	0	0	480	3840	—	—	120	60
" (2).....	0	0	480	480	—	—	60	240
" (3).....	0	0	120	0	—	—	120	60
" (4).....	0	0	1920	960	—	—	240	1920

* Dilution limit of distinct (+) agglutination.

— No titration.

Two "D" cultures, Rivers and No. 103, and four mucoid strains, Nos. 329, 544, DC 12, and DC 30, were compared. In addition to these, mucoid strains from the nasal passages of four stock rabbits affected with chronic snuffles were used, as well as serum from each of these animals. Direct and cross-agglutinations were made wherever possible and repeated a number of times. A control titration of the homologous strain and serum was included in each series of determinations.

The results of these several tests are summarized in Table VI. The two "D" antisera agglutinated the "G" variants of the "D" but none of the "G" variants of the mucoid strains to any significant

amount, while the four mucoid sera flocculated the six variant mucoid cultures, but practically none of the "D" variant strains. The four sera from the snuffles rabbits agglutinated the mucoid variant but not the "D" variant strains. The "D" variant cultures behaved similarly in the various sera; the mucoid variant cultures were also quite regular in this respect, except for No. 544 and snuffles (1), which appeared to flocculate more readily than the others.

These tests and similar ones on many other cultures show that "D" cultures differ from mucoid cultures in their antigenic properties but are themselves mutually related.[4]

A further consideration of the data presented will be given in the next paper of this series.

SUMMARY.

1. From 1922 to 1926, mucoid types of *Bact. lepisepticum* have proved more prevalent that "D" types in certain rabbit communities.

2. The bacteria from the mucoid colonies are larger than the "D" type; "G" variants are very small. All types are largest during the period of logarithmic growth. Differences in size appear to be determined chiefly by the amount of "cytoplasmic" substance.

3. The mucoid colony is larger and appears watery as compared with the "D" colony.

4. Fermentation tests, indole and nitrate reduction reactions of mucoid and "D" types and their variants are similar.

5. The acid agglutination zone of the mucoid type is similar to that of the "D" type.

6. The mucoid type is more stable in suspension than the "D" type.

7. The negative potential of mucoid types is similar to that of the "D" and higher than that of "G" variants.

8. The behavior of the mucoid type of bacilli in fluid media differs from that of the "D" strains in the following respects. About 500 or more mucoid organisms per cc., inoculated into plain broth, will grow; "D" strains require seedings of at least 100,000. A lag of 3 to 48 hours duration occurs in the growth of mucoid strains, according

[4] Recent publications by A. Tanaka, *J. Infect. Dis.*, 1926, xxxviii, 389 to 429, state that the antigenic properties of various strains of Pasteurella are similar. Apparently, he has experienced no difficulty in obtaining agglutination of his various strains; our experience and that of De Kruif have been quite the contrary.

to the amount of original seeding. This is considerably longer than that observed for the "D" strains. The period of multiplication is relatively long—24 to 48 hours—and the maximum number per cc. does not exceed 600,000,000. "D" strains reach 1,000,000,000 per cc. within 24 hours. "G" variants appear very slowly in mucoid cultures as contrasted with "D" strains, where their occurrence is rapid and abundant. The presence in the broth of blood or other oxygen-absorbing substances enables less than ten mucoid organisms per cc. to multiply without lag and to reach a maximum number of about 600,000,000 per cc. in 24 to 48 hours. "D" strains reach a count of 1,000,000,000 per cc. within 12 hours under similar conditions.

9. "D" and mucoid strains, inoculated into 750 cc. of plain or blood broth, and incubated at 37°, remain alive for more than 12 months. The numbers per cc. decrease to about 1/1000 of the maximum after the first 6 weeks. Subsequently, the rate of decrease is slower. The original strains are gradually replaced by "intermediate" and "G" colonies.

10. The various mucoid strains proved similar in their antigenic properties. "D" strains were also mutually related, but no cross-agglutination between "D" and mucoid types was demonstrable.

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BIOLOGY OF BACTERIUM LEPISEPTICUM.

IV. Virulence of Diffuse and Mucoid Types and Their Variants.

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In the preceding paper (1, *a*) we dealt with a number of physical, chemical, and biological characteristics of *Bact. lepisepticum;* in this present one we shall take up the question of virulence,—its relation to certain other bacterial characteristics and to the occurrence of the natural infection.

Virulence of "D" and Mucoid Types and Their Variants.

At the present time, there is but one method of estimating the virulence of a microorganism,—that of animal inoculation. The technique is to administer the specific microbe to a number of laboratory animals, and to observe their subsequent behavior over a fixed period of time. The effects produced are usually recorded in terms of the duration of life of the animals inoculated, but under some conditions, in those of the number of animals that become infected or in types and severity of infection. The investigator's interpretation of such results depends upon his attitude toward certain complicating factors. Chief among these is the difference in reaction of the individual animals to the microorganism. If a group of animals is injected at one time with the same dose of pathogenic bacteria, certain ones may die, others survive with signs of chronic infection, and still others prove refractory. Those that die may succumb after different intervals of time, and those that survive show varying types and degrees of infection. Thus it is evident that the method of titrating virulence by means of animal inoculation is essentially an attempt to evaluate an unknown in terms of an arbitrary standard, which is itself ill defined and subject to wide variation.

Two possible explanations for the variability of animal reaction as just illustrated must be considered, namely, that it is due either to chance or to actual differences in the resistance of each individual. Topley (2) and Lockhart (3), on the basis of statistical analyses,—which we believe to be incorrectly applied—assume that the potential reaction of each animal is the same and is constant, variations in their behavior being due entirely to chance. We, on the other hand, are convinced as result of the pathological and bacteriological evidence obtained through the observation of experimental mouse typhoid (1, *b*, *c*) and rabbit septicemia infections (1, *d*) that animals react differently to bacteria because of differences in their resistance, that those which die soon after injection do so because they are relatively susceptible, and that those which escape infection do so because they are especially resistant. Consequently, until direct evidence to the contrary is forthcoming, we shall regard the reaction of the host to the presence of injurious influences as a standard of measurement which varies in each animal according to its resistance.

As one comes to recognize that the amount of resistance differs among individuals (1, *b*, *c*, *d*), races (4), and at seasons of the year (5), that it is affected by diet (1, *e*), light, and other environmental conditions, and consequently that in a virulence titration this host reaction, which we take as the standard of measurement, reflects differences in animal susceptibility as well as bacterial potentialities, one comes also to appreciate the necessity of rigid measures of control during experimentation. As many variables as possible must be eliminated. An inbred race of animals (1) should be used, whose (2) environmental conditions have been uniform and such that they have not been exposed (3) to the specific organism. As large numbers of them as possible (4) of similar age and weight should be employed for each titration. Dosage should be measured in terms of numbers of organisms given to each animal. And finally, if the true, or native, pathogenicity of a microbe under natural conditions is to be determined, the native host should be employed and the bacteria administered by way of the normal portal of entry.

Unfortunately, experimental methods are governed to no small extent by working facilities. Thus, while our own virulence determinations of mouse typhoid bacilli have been carried out under con-

ditions such as have been just outlined (1, *b*), the titrations of rabbit septicemia organisms described in the present paper have been limited by the small numbers of rabbits available and by the fact that they were not strain-inbred. We have used animals in groups of 8 to 10, of similar age and weight, bred at the Rockefeller Institute, not previously exposed to *Bact. lepisepticum;* and we have instilled intranasally a known number of organisms. This procedure has been followed by results which have been relatively constant and which indicate, we believe, the true or natural virulence of different strains of *Bact. lepisepticum.* Furthermore, the results have served as controls in estimating the reliability of other titration methods, less accurate, but more practical. Thus it was found that when a culture given intranasally to rabbits caused a high percentage of pneumonia and septicemia cases, it likewise proved fatal when injected in small numbers intrapleurally into rabbits, or intraperitoneally into mice, and conversely that strains showing little effect when given to rabbits by the normal portal of entry were less apt to kill when introduced parenterally. Hence these latter procedures were used to gain presumptive evidence of differences in the pathogenicity of a large number of strains of *Bact. lepisepticum* over varying periods of time.

Intranasal Inoculation of Rabbits.

In 1921, De Kruif (6) reported that "D" strains of *Bact. lepisepticum* are more virulent than their "G" variants, that the virulence of the "D" type is relatively constant, but that Type "G" virulence may be somewhat increased by rabbit passage. Since that time, we have compared the virulence of many "D" and mucoid strains and their variants, isolated from nasal carrier rabbits and from rabbits with abscesses, snuffles, otitis, pneumonia, and septicemia.

The results of a number of what we shall call "natural" virulence titrations of "D," mucoid, and "G" variant strains are summarized in Table I.

In each instance, 250,000,000 organisms of a 17 hour blood broth culture from the stock agar slant were instilled into the right and left nares of rabbits of similar age—5 months—and weight—800 to 1000 gm.—from the Rockefeller Institute breeding room. The Rivers "D" strain was isolated in 1923 from a rabbit which

had died from spontaneous septicemia. The "G" variant was obtained by cultivating the "D" strain 72 hours in plain broth (1, *g*). The mucoid strains were freshly isolated in 1925 from snuffles and nasal carrier rabbits (1, *d*).

Eleven titrations of the Rivers "D" strain have been made over a period of more than 2 years. In nine instances the mortality from

TABLE I.

Virulence of "D," Mucoid, and "G" Strains of Bact. lepisepticum Instilled Intranasally into Rabbits.

Strain.	Experiment.	Date.	Total No. used in experiment.	Pneumonia and septicemia.		Snuffles.		Carriers.		Resistant.	
				No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
Rivers "D"	1	1923	6	2	33.2	4	66.4	0	—	0	—
"	2	1923	6	1	16.6	5	83.4	0	—	0	—
"	3	1923	6	2	33.2	2	33.2	1	16.6	1	16.6
"	4	1924	6	3	50	0	—	1	16.6	2	33.4
"	5	1924	12	3	25	8	66.7	1	8.3	0	—
"	6	1924	12	8	66.7	2	16.6	0	—	2	16.6
"	7	1924	7	1	14.3	1	14.3	4	57.2	1	14.3
"	8	1925	11	3	27	6	54	1	9	1	9
"	9	1925	19	2	10	11	55	2	10	4	20
"	10	1925	5	1	20	2	40	2	40	0	—
"	11	1925	8	2	25	2	25	1	12.5	3	37.5
Total average, Rivers "D"			98	28	28.5	43	44	13	13.3	14	14
Rivers "G"	1	1925	11	0	—	0	—	0	—	11	100
Mucoid 544	1	1925	10	0	—	2	20	6	60	2	20
" 329	1	1925	10	0	—	4	40	5	50	1	10
" DC 30	1	1925	8	0	—	3	37.5	3	37.5	2	25
Total average			28	0	—	9	32.3	14	50	5	17.5

pneumonia and septicemia was between 10 and 33 per cent; in two, over 50 per cent. The average mortality was 28.5 per cent; chronic snuffles developed in an average of 44 per cent, and 13.3 per cent became chronic nasal carriers of the organisms. Two other "D" strains

from cases of spontaneous pneumonia showed a similar and apparently constant degree of virulence.[1]

The titration of the Rivers "G" strain included in Table I indicates the almost total lack of what we term "natural" virulence of this variant. None of the 11 rabbits used in the test became infected.

Typical intranasal titrations of three mucoid strains are also shown in Table I. None of the animals died; 20 to 40 per cent, average 32.3 per cent, developed chronic snuffles, and 37.5 to 60 per cent, average 50 per cent, became chronic carriers of the bacteria.

These results show the relative degree of virulence of "D" and mucoid types of *Bact. lepisepticum* and their variants under natural conditions of infection, indicating besides that the "D" type tends

TABLE II.

Virulence of "D," Mucoid, and "G" Types of Bact. lepisepticum Injected Intrapleurally into 800 to 1000 Gm. Rabbits.

Type.	No. of organisms injected.				
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
"D"	†2 days.	3	5	8	12±*
Mucoid	6	6	8	10±*	
"G"	6	6	10±*	10±*	

† Duration of life in days after injection.

* About 50 per cent of the animals survived. An average value indicating duration of life was difficult to obtain.

either to kill its host or to induce chronic snuffles, that the mucoid type is relatively non-lethal, but induces snuffles and carrier cases, while the "G" variant is practically without effect. Furthermore, they furnish evidence that the virulence of different strains of the same type is relatively speaking the same and that it is constant under natural conditions.

Intrapleural Inoculation of Rabbits.

Rabbits similar in age and weight to those of the preceding series were employed. The cultures were taken from stock blood agar

[1] Two illustrative titrations of Strain 103, Type "D," are described in full in the *J. Exp. Med.*, 1926, xliii, 573.

TABLE III.

Virulence of "D" and "G" Types of Bact. lepisepticum Injected Intraperitoneally into Mice.

Strain.	Date.	Average No. of bacteria injected.							
		10^8	10^7	10^6	10^5	10^4	10^3	10^2	10^1
Rivers "D"	1/14/24	1-*			2-2		2-2		3-3
"	1/ 7/24							2-S.	
"	1/ 9/24	1-1					1-2-S.-S.		4-S.-S.-S.
"	1/15/24		1-			1-1		1-S.	1-1
"	1/25/24				1-1-1-1-	1-1-	1-1-1-	1-1-2-	
					1-1-1-1-	1-1-1-	1-1-1-	3-3-3-	
					1-S.	1-1-1-	1-1-1-	3-3-3-	
						3-3-4-	2-2-2-	3-4-4-	
						S.-S.-S.	2-3-4-	4-5-S.-	
							S.-S.	S.-S.-S.	
"	2/ 5/24	1-1		1-1		2-3		1	
"	3/ 5/24			1-1		1-1		2-2	
"	4/ 5/24			1-1		S.-S.		S.-S.	S.-S.
"	12/12/24				1-				1-2
"	1/ 3/25		1-1				1-S.		
"	1/13/25			1-1			S.-S.		
"	1/30/25			1-1			1-1		
"	2/ 7/25		1-1		1-3				
"	2/17/25		1-1		1				
"	3/ 4/25		1-1		S.-S.				
"	3/10/25		1-1		S.-S.				
"	4/ 7/25		1-1		S.-S.				
"	4/17/25		1-1		1-1				
"	5/ 7/25		1-1		1-1				

"	6/30/25		1-1		3-3				
"	8/20/25		1-1			8-S.			
"	11/18/25	1-1	1-2			S.-S.			
"	11/19/25	1-1	1-2			S.-S.			
"	11/20/25	1-1	2-2			4-S.			
"	12/ 9/25	1-1		1-1			1-S.		
"	12/15/25	1-1	1-2				2-S.		
Average.....		1 day.	1.2 days.	1 day.	1.4 days: 24% S.	2 days: 37% S.	1.6 days: 37% S.	2.6 days: 30% S.	2.1 days: 41% S.
203 "D"	2/ 5/24	1-1		2-3		2-S.		2-1	
115 "D"	2/ 5/24	1		1-1		2-2		2-3	
Rivers "G"	1/ 7/24		S.–S.			S.-S.		S.-S.	
"	1/10/24	1-1	2						
"	1/15/24	1	1–S.	S.-S.	S.–S.				
Average.....		1	1.5–60% S.	S.	S.	S.		S.	
88 "G"	2/ 5/24	1-S.		S.-S.		S.-S.		S.-S.	
157 "G"	2/ 5/24	1-1		S.-S.		S.-S.		S.-S.	
188 "G"	2/ 5/24	1-1		S.-S.		S.-S.		S.-S.	
79 "G"	2/ 5/24	1-1		S.-S.		S.-S.		S.-S.	
186 "G"	2/ 5/24	1-S.		S.-S.		S.-S.		S.-S.	

* Numerals indicate duration of life in days after injection.
S. = Survived 30 days.

slants, grown overnight in blood broth, and injected into the right chest cavity. Bacterial counts were made by the dilution method.

In Table II are summarized the effects of twelve titrations of "D" strains on 96 rabbits, six titrations of "G" strains on 30 rabbits, and six titrations of mucoid strains on 48 rabbits. Although less striking, the results are similar to those obtained from the nasal instillation of the cultures. "D" strains killed in the shortest period of time, mucoid strains were less effective, while "G" strains proved least virulent. Different strains of the same type behaved similarly; no profound fluctuation in virulence of any type-pure strain was observed.

Intraperitoneal Inoculation of Mice.

We have pointed out that a number of titrations on freshly isolated strains and strains used over long periods of time were made by the method involving the intraperitoneal injection of mice. A 17 hour blood broth culture was suitably diluted, counted, and then injected intraperitoneally into mice 12 to 14 weeks old, from the Rockefeller Institute breeding room. Whenever these freshly isolated strains were tested, the Rivers "D" stock culture was titrated simultaneously as a control.

Table III summarizes the titrations of "D" and "G" strains. The Rivers "D" strain has been tested twenty-six times in this manner over a period of 2 years. No significant fluctuations in virulence are apparent. 1 million organisms or more killed mice regularly within 24 hours; 100,000 in 1.4 days on the average, with 24 per cent survivors; smaller doses acted accordingly. Two other "D" cultures showed similar high virulence. The Rivers "G" variant and four other "G" strains failed to kill when less than 10,000,000 organisms were given. No change in the virulence of these strains was observed.

58 titrations of 36 mucoid strains are grouped chronologically in Table IV. The results of the titrations of the Rivers "D" strain, carried out simultaneously with each series of mucoid strains as a control, are omitted from this table and grouped in Table III. They may be identified by comparing the respective dates of each experiment.

The eight mucoid cultures designated by capital letters came from snuffles, carrier, and pneumonia rabbits at a farm in New City, N. Y. The *lepisepticum*

TABLE IV.

Virulence of Mucoid Strains of Bact. lepisepticum Injected Intraperitoneally into Mice.

Strain.	No. of organisms injected.						
	Date.	10^7	10^6	10^5	10^4	10^3	10^2
BBB 24	12/17/24			S.-S.			3-S.
DC 30	12/17/24			S.-S.			5-S.
DC 66	12/17/24			3-S.			S.-S.
590	1/ 3/25		S.-S.			S.-S.	
652	1/ 3/25			S.-S.			S.-S.
560 (1)	1/ 3/25		S.-S.			S.-S.	
560 (2)	2/ 7/25		S.-S.			1-3	
560 (3)	4/17/25		1-2			S.-S.	
545	1/13/25		S.-S.			S.-S.	
569 (1)	1/13/25		S.-S.			4-5	
569 (2)	2/ 7/25		1			3-3	
569 (3)	6/30/25			1-1		S.-S.	
544 (1)	1/13/25		S.-S.			S.-S.	
544 (2)	1/30/25		S.-S.			S.-S.	
544 (3)	2/17/25		S.-S.			S.-S.	
544 (4)	3/10/25		S.-S.			S.-S.	
544 Rab (5)	3/10/25		S.-S.			S.-S.	
329 (1)	2/ 7/25		1-1			1-3	
329 (2)	3/10/25		4-S.			3-4	
329 Rab (3)	3/10/25		1-1			4-4	
329 (4)	11/20/25		3-4		S.-S.		S.-S.
321 (1)	2/17/25		2-S.			1	
321 (2)	11/18/25	1-1		2-S.		4-S.	
375	2/17/25	1-1				3-S.	
DC 47	3/ 4/25		2-2			S.-S.	
DC 335	3/ 4/25	1-1			S.-S.		
DC 672	3/ 4/25		S.-S.			S.-S.	
BC 7	3/ 4/25		S.-S.			S.-S.	
DC 72	3/ 4/25		4-S.			2-S.	
536	4/ 7/25		S.-S.			S.-S.	
543	4/ 7/25		S.-S.			S.-S.	
Smith (1)	4/ 7/25		1-S.			S.-S.	
541 (1)	4/17/25			S.-S.			S.-S.
541 (2)	11/19/25	1-1	3-S.		S.-S.		
81	4/17/25		S.-S.			S.-S.	
Smith (72)	5/ 7/25		1-S.			S.-S.	
577	6/30/25		1-1				S.-S.
557 (1)	6/30/25		1-1				S.-S.
557 (2)	11/19/25	1-1	4-4		2-4		
557 (3)	12/ 9/25	S.-S.		S.-S.			S.-S.
485 (1)	6/30/25		1-1				S.-S.

TABLE IV—*Concluded.*

Strain.	No. of organisms injected.						
	Date.	10^7	10^6	10^5	10^4	10^3	10^2
485 (2)	11/19/25	1-1	S.-S.		S.-S.		
485 (3)	12/ 9/25	1-2	2-S.			2-3	
399 (1)	6/30/25			1-1			S.-S.
399 (2)	11/20/25	1-1		3-4			3-4
399 (3)	12/ 9/25	1-2		S.-S.			2-2
587 (1)	8/20/25	1-1			8-S.		
587 (2)	11/19/25	1-1	4-4		2-4		
555 (1)	11/19/25	2-3		S.-S.		S.-S.	
555 (2)	12/ 9/25	1-S.		S.-S.			3-S.
492	11/18/25	1-S.	S.-S.		S.-S.		
369	11/18/25	1-2	2-S.		S.-S.		
556	11/18/25	S.-S.	S.-S.		S.-S.		
349	11/18/25	1-1	2-3		S.-S.		
395 (1)	11/20/25		1-1		S.-S.		
395 (2)	12/ 9/25	1-1-2-S.	S.-S.-S.-S.	S.-S.		2-S.	3-S.
338	11/20/25	2-2		S-S.			
516	12/15/25	1-2-2-2-2-4		2-S.-S.-S.-S.-S.			S.-S.-S.-S.-S.-S.
Total average.....		1.4 days: 14.5% S.	2 days: 60% S.	1.7 days: 75% S.	4 days: 80% S.	2.9 days: 69% S.	2.8 days: 75% S.

S. = Survived 30 days.
Numerals = Duration of life in days after injection.

infection is endemic there among a stock of about 2500 rabbits. Strains designated as "Smith 1" and "72" came from cases of pneumonia in a spontaneous epidemic among a group of rabbits at Ray Brook, N. Y., and were kindly supplied by Dr. D. T. Smith. Strains numbered in the three, four, and five hundreds came from a special group of rabbits at the Rockefeller Institute. The source and treatment of these rabbits were as follows. 60 animals from the Institute breeding room, not previously exposed to *Bact. lepisepticum* infection, were placed in separate cages in a small, special room, in December, 1924. Caretakers and attendants were allowed free access, so that infection from the other Rockefeller Institute stock rabbits might be introduced. Nasal swab cultures were taken at frequent intervals and the types of organisms present were studied. Some of the virulence titrations on the mucoid strains recovered in this way have been included in this and the following tables.

The data in Table IV bring out one fact clearly, namely, that the mucoid type of *Bact. lepisepticum* is consistently less virulent for mice than the "D" and slightly more virulent than the "G" type. 1 million mucoid organisms killed an average of 40 per cent of the animals in 2 days and failed entirely to kill 60 per cent. Smaller doses were without effect in more than 68 per cent of the animals. Furthermore, the table indicates that mucoid strains, living in the nasal tissues of rabbits, do not change in pathogenicity; cultures presumably of the same mucoid strains obtained on two, three, or four different occasions from Rabbits 560, 569, 544, 329, 321, 541, 557, 485, 399, and 587 proved to be similar in virulence. Although no significant differences in the virulence of these strains were apparent, it seemed wise to test this question further by the following method.

A series of "intraperitoneal titrations" upon mice was carried out with twenty-seven mucoid strains recovered from the nasal passages of rabbits in the special room at the Rockefeller Institute, referred to above. Cultures obtained from rabbits within 4 days of the time of the experiment were given intraperitoneally to 12 mice, in the following dilutions: 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-5}. The Rivers "D" strain, the virulence of which is known, was administered each day to a similar group as a control. The number of organisms given to each mouse was determined by the plating method. Duration of life was noted and the healthy survivors discarded after 14 days.

The results of these tests are recorded in Table V, and are rearranged in Table VI to show the average duration of life of mice receiving similar doses of different strains. In spite of every precaution, irregularities in survival time occurred. Some cultures which seemed to show a relatively great killing power in one dilution were less active in other dilutions. Frequently, 1 of the 3 mice given a certain dose of culture survived over a period of time out of all proportion to that of its mates. These irregularities could not have been due to microbic factors, and it is hardly possible that chance factors alone would lead to such discrepancies. We believe the inconsistencies to be due to differences in the resistance of the mice, and accordingly that the significance of the results must be determined by inspection alone.

Clearly, the Rivers "D" strain is more virulent than the mucoid cultures; it killed the mice in the shortest period of time in various doses. Furthermore, Cultures 518, 560, 555, 512, and 369, which

TABLE V.

"Intraperitoneal Titrations" of Mucoid Strains in Mice.

Culture.	Dilution.	No. of organisms injected.	Duration of life in days for the 3 mice.	Average duration of life.	Average No. of survivors.
3/23/26 Rivers.	10^{-1}	104,000,000	0.7-0.7-0.7	0.7	
"	10^{-2}	10,400,000	0.7-0.7-2	1.1	
	10^{-3}	1,040,000	0.7-0.7-2	1.1	
	10^{-5}	10,400	2-7-S.	4.5	1
563	10^{-1}	60,800,000	0.7-0.7-4	1.8	
	10^{-2}	6,080,000	2-2-S.	2.0	1
	10^{-3}	608,000	4-S.-S.	4.0	2
	10^{-5}	6,080	3-6-S.	4.5	1
518	10^{-1}	105,000,000	0.7-0.7-5	2.1	
	10^{-2}	10,500,000	0.7-2-2	1.6	
	10^{-3}	1,050,000	2-2-5	3.0	
	10^{-5}	10,500	2-2-4	2.6	
330	10^{-1}	130,800,000	0.7-0.7-2	1.1	
	10^{-2}	13,080,000	0.7-2-S.	1.3	1
	10^{-3}	1,308,000	2-S.-S.	2.0	2
	10^{-5}	13,080	S.-S.-S.		3
329	10^{-1}	68,400,000	0.7-0.7-2	1.1	
	10^{-2}	6,840,000	4-6-S.	5.0	1
	10^{-3}	684,000	5-6-S.	5.5	1
	10^{-5}	6,840	2-5-S.	3.5	1
379	10^{-1}	87,000,000	0.7-2-3	1.9	
	10^{-2}	8,700,000	2-8-S.	5.0	1
	10^{-3}	870,000	1-4-11	5.0	
	10^{-5}	8,700	8-10-13	10.0	
3/24/26 Rivers.	10^{-1}	105,000,000	0.7-0.7-1	0.8	
	10^{-2}	10,500,000	0.7-1-6	2.6	
	10^{-3}	1,050,000	2-6	4.0	
	10^{-5}	10,500	0.7-6-S.	6.5	1
565	10^{-1}	72,000,000	1-3-4	2.6	
	10^{-2}	7,200,000	0.7-2-4	2.2	
	10^{-3}	720,000	0.7-S.-S.	0.7	2
	10^{-5}	7,200	1-2-3	2.0	

TABLE V—*Continued.*

Culture.	Dilution.	No. of organisms injected.	Duration of life in days for the 3 mice.	Average duration of life.	Average No. of survivors.
555	10^{-1}	68,400,000	0.7-0.7-3	1.6	
	10^{-2}	6,840,000	1-2-3	2.0	
	10^{-3}	684,000	0.7-2-S.	0.9	1
	10^{-5}	6,840	3-S.-S.	3.0	2
462	10^{-1}	120,000,000	0.7-0.7-3	1.5	
	10^{-2}	12,000,000	0.7-1-4	1.9	
	10^{-3}	1,200,000	2-3-S.	2.5	1
	10^{-5}	12,000	2-2-S.	2.0	1
395	10^{-1}	84,000,000	2-4-S.	3.0	1
	10^{-2}	8,400,000	2-5-S.	3.5	1
	10^{-3}	840,000	5-6-S.	5.5	1
	10^{-5}	8,400	6-13-S.	9.5	1
544	10^{-1}	42,000,000	0.7-1-4	1.9	
	10^{-2}	4,200,000	0.7-7-2	1.1	
	10^{-3}	420,000	3-S.-S.	3.0	2
	10^{-5}	4,200	3-13-S.	9.5	1
543	10^{-1}	46,800,000	S.-S.-S.		3
	10^{-2}	4,680,000	3-S.-S.	3.0	2
	10^{-3}	468,000	5-S.-S.	5.0	2
	10^{-5}	4,680	S.-S.-S.		3
556 A	10^{-1}	117,000,000	0.7-3-4	2.6	
	10^{-2}	11,700,000	1-3-5	3.0	
	10^{-3}	1,170,000	1-5-S.	2.0	1
	10^{-5}	11,700	3-3-5	3.6	
3/25 Rivers.	10^{-1}	120,000,000	0.7-0.7-0.7	0.7	
	10^{-2}	12,000,000	2-2-S.	2.0	1
	10^{-3}	1,200,000	2-2-2	2.0	
	10^{-5}	12,000	2-3-3	2.6	
512	10^{-1}	69,000,000	0.7-2-2	1.6	
	10^{-2}	6,900,000	0.7-2-6	2.9	
	10^{-3}	690,000	2-3-6	3.6	
	10^{-5}	6,900	2-4-1-S.	3.0	1
557	10^{-1}	76,800,000	0.7-2-S.	1.3	1
	10^{-2}	7,680,000	S.-S.-S.		3

TABLE V—*Continued.*

Culture.	Dilution.	No. of organisms injected.	Duration of life in days for the 3 mice.	Average duration of life.	Average No. of survivors.
557	10^{-3}	768,000	S.-S.-S.		3
	10^{-5}	7,680	S.-S.-S.		3
383	10^{-1}	78,000,000	4-5-S.	4.5	1
	10^{-2}	7,800,000	S.-S.-S.		3
	10^{-3}	780,000	4-S.-S.	4.0	2
	10^{-5}	7,800	6-9-S.	7.5	1
593	10^{-1}	147,000,000	2-2-2	2.0	
	10^{-2}	14,700,000	4-4-S.	4.0	1
	10^{-3}	1,470,000	3-3-5	3.6	
	10^{-5}	14,700	5-7-S.	6.0	1
375	10^{-1}	120,000,000	0.7-0.7-2	1.1	
	10^{-2}	12,000,000	2-4-S.	3.0	1
	10^{-3}	1,200,000	0.7-3-3	2.2	
	10^{-5}	12,000	6-7-S.	6.5	1
368	10^{-1}	103,800,000	0.7-3-3	2.2	
	10^{-2}	10,380,000	3-6-S.	4.5	1
	10^{-3}	1,038,000	4-S.-S.	4.0	2
	10^{-5}	10,380	9-9-S.	9.0	1
346	10^{-1}	75,000,000	4-4-S.	4.0	1
	10^{-2}	7,500,000	3-S.-S.	3.0	2
	10^{-3}	750,000	5-S.-S.	5.0	2
	10^{-5}	7,500	S.-S.-S.		3
3/26 Rivers.	10^{-1}	156,000,000	0.7-0.7-0.7	0.7	
	10^{-2}	15,600,000	0.7-2-2	1.6	
	10^{-3}	1,560,000	2-2-2	2.0	
	10^{-5}	15,600	2-3-S.	2.5	1
535	10^{-1}	70,400,000	0.7-2-2	1.2	
	10^{-2}	7,040,000	2-3-4	3.0	
	10^{-3}	704,000	2-S.-S.	2.0	2
	10^{-5}	7,040	8-S.-S.	8.0	2
492	10^{-1}	162,000,000	2-5-6	4.3	
	10^{-2}	16,200,000	2-5-7	4.6	
	10^{-3}	1,620,000	S.-S.-S.		3
	10^{-5}	16,200	2-S.-S.	2.0	2

TABLE V—*Concluded.*

Culture.	Dilution.	No. of organisms injected.	Duration of life in days for the 3 mice.	Average duration of life.	Average No. of survivors.
560	10^{-1}	51,600,000	0.7-2-2	1.6	
	10^{-2}	5,160,000	2-3-4	3.0	
	10^{-3}	516,000	2-3-5	3.3	
	10^{-5}	5,160	2-2-3	2.3	
369	10^{-1}	40,000,000	0.7-0.7-2	1.1	
	10^{-2}	4,000,000	2-5-7	3.6	
	10^{-3}	400,000	2-5-S.	2.3	1
	10^{-5}	4,000	5-7-S.	6.0	1
587	10^{-1}	42,000,000	S.-S.-S.		3
	10^{-2}	4,200,000	S.-S.-S.		3
	10^{-3}	420,000	S.-S.-S.		3
	10^{-5}	4,200	2-S.-S.	2.0	2
349	10^{-1}	39,000,000	3-3-3	3.0	
	10^{-2}	3,900,000	2-4-S.	3.0	1
	10^{-3}	390,000	S.-S.-S.	3-S.	3
	10^{-5}	3,900	2-S.-S.	2.0	2
368	10^{-1}	51,600,000	4-4-S.	4.0	1
	10^{-2}	5,160,000	2-3-5	3.3	
	10^{-3}	516,000	4-10-S.	7.0	1
	10^{-5}	5,160	3-3-S.	3.0	1
183	10^{-1}	40,000,000	2-2-2	2.0	
	10^{-2}	4,000,000	2-3-5	3.3	
	10^{-3}	400,000	2-3-3	2.6	
	10^{-5}	4,000	3-5-11	6.3	

S. = Surviving and clinically healthy at 14 days.

proved lethal within 1 to 3 days when given in doses of 10^7 to 10^5, and to some of the mice in doses as small as 10^3, would seem from this experiment to be more pathogenic than Cultures 587, 557, 492, 346, and 349, which, in doses of 10^7 to 10^5 killed usually after 3 days and in smaller doses were generally without effect. Nevertheless, we wished to gain further evidence of these differences by more direct methods. We therefore chose an apparently virulent mucoid strain, No. 518, another less so, No. 369, and a third of little virulence, No. 383 as

TABLE VI.

Average Duration of Life of Mice Receiving Similar Doses of Different Mucoid Strains of Bact. lepisepticum.

Culture.	10^8		10^7		10^6		10^5		10^4		10^3	
	Duration of life in days.	No. survivors.	Duration of life in days.	No. survivors.	Duration of life in days.	No. survivors.	Duration of life in days.	No. survivors.	Duration of life in days.	No. survivors.	Duration of life in days.	No. survivors.
Rivers.	0.7, 0.8, 0.7, 0.7: = 0.7 av.		1.1, 2.6, 2, 1.6 = 1.4 av.	1	1.1, 4, 2, 2, = 2.3 av.				2.6, 2.5, 4.5, 6.5 = 4.0 av.	3 = 0.7 av.		
518	2.1		1.6		3.0				2.6			
560			1.6		3.0		3.3				2.3	
555			1.6		2.0		0.9	1			3.0	2
512			1.6		2.9		3.6				3.0	1
369			1.1		3.6		2.3	1			6.0	1
544			1.9		1.1		3.0	2			9.5	1
462	1.5		1.9		2.5	1			2.0	1		
535			1.2		3.0		2.0	2			8.0	2
563			1.8		2.0	1	4.0	2			4.5	1
329			1.1		5.0	1	5.5	1			3.5	1
379			1.9		5.0	1	5.0				10.0	
565			2.6		2.2		0.7	2			2.0	
183			2.0		3.3		2.6				6.3	
556	2.6		3.0		2.0	1			3.6			
375	1.1		3.0	1	2.2				6.5	1		
593	2.0		4.0	1	3.6				6.0	1		
395			3.0	1	3.5	1	5.5	1			9.5	1
368			4.0	1	3.3		7.0	1			3.0	1
330	1.1		1.3	1	2.0	2			—	3		

543			—	3	3.0	2	5.0	2		—	3
557			1.3	1	—	3	—	3		—	3
383			4.5		—	3	4.0	2		7.5	1
368	2.2		4.5	1	4.0	2			9.0	1	
346			4.0	1	3.0	2	5.0	2		—	3
492	4.3		4.6		—	3			2.0	2	
587			3.0		3.0	1	—	3		2.0	2
349			—	3	—	3	—	3		2.0	2

av. = average.

determined by the intraperitoneal method, and instilled a 24 hour blood broth culture of each into the nares of ten 600 to 800 gm. rabbits from the Rockefeller Institute breeding room. About 125,000,000 organisms were introduced into each naris. The results of this test are shown in Table VII.

3 weeks after infecting the animals, the carrier rate and percentage of resistant animals in each group were similar. 2 animals in the 369 series, however, died from pleuropneumonia. This relationship still obtained after 6 and 9 weeks; hence we concluded that these strains showed about the same degree of natural virulence for rabbits, and that they were similar to the other cultures studied (Table I).

TABLE VII.

Virulence of Mucoid Strains of Bact. lepisepticum Instilled Intranasally into Rabbits.

Date.	Group.	Specific mortality.	Snuffles.	Carriers.	Refractory animals.
		per cent	*per cent*	*per cent*	*per cent*
4/4/26 infected.					
4/24/26	383	0	11.1	67	33
"	369	20	0.0	75	25
"	518	0	12.5	62	38

Effect of Artificial Cultivation and of Animal Passage on "D," Mucoid, and "G" Types.

In the experiments so far described, we have noted several times that the virulence of "D," mucoid, or "G" types does not fluctuate significantly. That the virulent "D" organism gives off less virulent "G" variants was observed *in vitro* by De Kruif (6) and by us in experiments involving infection of the nasal passages of rabbits (1, *d*). But nothing has been noted up to the present that would indicate the occurrence of the reverse process, namely, the change from a less virulent to a more virulent type. We undertook to follow any type changes which might occur, first, by growing the bacteria in various artificial media, and second, by making animal passages.

Artificial Cultivation.—Since the change from the virulent "D" or mucoid type to the avirulent "G" variant is brought about when the oxygen tension of the media is high, and inhibited when it is low (1,

g), it seemed desirable to ascertain whether "G" or mucoid types may change to more virulent forms when grown in media of low oxygen tension. Hence, a number of strains was seeded into plain broth and into broth enriched with yeast extract, vegetable, or animal tissue, in which the oxygen tension was reduced mechanically, or by the addition of blood, potato, iron salts, and oxides (2). Rapid and slow passages were tried at different temperatures, but the results were entirely negative. Neither type specificity nor grade of virulence was altered.

Animal Passage.—Experiments 1 to 5 were planned to test the effect, if any, of direct passages through the rabbit, without artificial cultivation, of types of *Bact. lepisepticum* introduced into the body in an abnormal way.

TABLE VIII.

Direct Intrapleural Rabbit Passage of Bact. lepisepticum, "D" Type, R. "63."

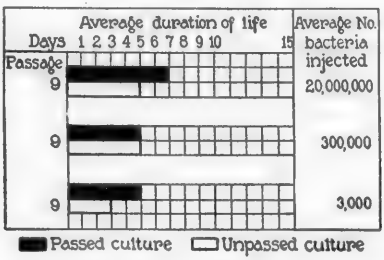
Rabbit No.	No. of passages.	Culture injected.	Quantity injected.	Duration of life in days after injection.
			cc.	
1	1	18 hr. blood broth.	1	2
1 A		18 " " "	1	5
2	2	Pleural exudate: Rabbit 1.	1	7
3	3	" " " 2.	1	5
4	4	" " " 3.	1	5
5	5	" " " 4.	1	1
6	6	" " " 5.	1	1
7	7	" " " 6.	½	2
8	8	" " " 7.	½	3

Experiment 1.—A culture of *Bact. lepisepticum*, Type "D," R. 63, obtained from the nasal passages of a rabbit at the onset of spontaneous snuffles, was grown for 18 hours in rabbit blood broth, and injected in 1 cc. amounts into the left pleural cavity of two 800 to 1000 gm. rabbits. 2 days later 1 rabbit was dead; on the 5th day the second rabbit died. 1 cc. of the pleural exudate of the first rabbit was injected directly into the left pleural cavity of a third rabbit of the same size. 7 days later this rabbit died, and 1 cc. of its pleural exudate was injected as before into a fourth 800 gm. rabbit. This procedure was continued for 8 direct rabbit passages. The results, summarized in Table VIII, indicate that the duration of life decreased somewhat with the intrapleural passage of the culture.

The pleural exudate was then taken from Rabbit "8" and injected directly, without artificial cultivation, intrapleurally into 4 rabbits in the following broth dilutions: 1:1, 1:100, 1:10,000, and 1:1,000,000. ½ cc. of the undiluted pleural

exudate was also instilled into the nares of each of 4 rabbits. At the same time, a 6 hour rabbit blood broth culture from the original, unpassed stock culture was injected intrapleurally into 4 rabbits in 1 cc. quantities, undiluted, 1:10, 1:100, and 1:1000; and 4 rabbits were given the undiluted culture intranasally in doses of ½ cc. The actual number of bacteria per cc. in each culture was determined by the plating method.

None of the rabbits inoculated intranasally showed clinical signs of infection. Those injected intrapleurally, however, died at intervals of 5 to 7 days (Text-fig. 1). And the average duration of life of animals receiving the "passed" culture was at least as long as those injected with the "unpassed" culture. It is concluded, therefore, that under the conditions of this experiment, 9 pleural passages did not affect the intrapleural or intranasal virulence of the "D" strain, R. 63, of *Bact. lepisepticum.*



TEXT-FIG. 1. Virulence of 9 passage culture and "unpassed" stock culture of *Bact. lepisepticum.* "D" strain, R. 63.

"Average number bacteria injected" represents the mean of the nearest comparable figures.

Experiment 2.—Another virulent "D" strain, Rivers, previously described, was grown 17 hours in rabbit blood broth. 1 cc. was then injected intrapleurally into a 1000 gm. rabbit. This animal died the following day. A heart's blood broth culture from this rabbit, incubated 14 hours, was injected intrapleurally into 6 rabbits in the following dilutions: 1:1, 1:10, 1:1000, and 1:100,000. The number of bacteria per cc. was estimated by the usual plating method. 6 additional rabbits were injected intranasally with the undiluted culture.

2 animals receiving the nasal injections died with pleuropneumonia; 4 of the 6 developed snuffles; 2 remained well. Of those receiving the culture intrapleurally 1, with the 600,000,000 dose, died in 1 day; 1, with 60,000,000, in 1 day; 2, with 600,000, in 2 days; and 2, with 6000, in 1 and 2 days.

1 cc. of the pleural fluid of the first rabbit to die was injected intrapleurally into a 1000 gm. rabbit, and the number of bacteria thus injected was determined as before. A similar direct pleural passage was continued through 7 rabbits with the

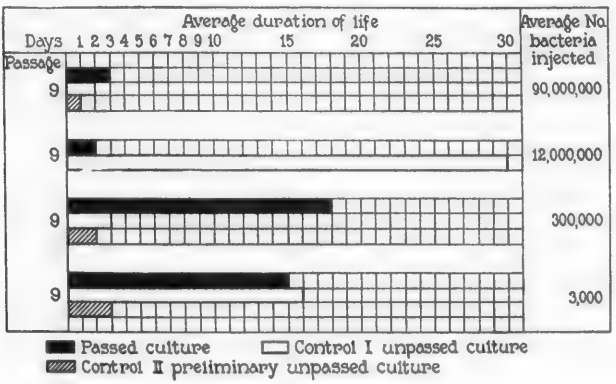
result that most of the rabbits received over a billion organisms and were dead within 24 to 48 hours (Table IX).

The pleural exudate of the rabbit of the eighth passage was then injected intrapleurally into 6 rabbits in the following dilutions: 1:100, 1:1000, 1:100,000,

TABLE IX.

Direct Intrapleural Rabbit Passage of Bact. lepisepticum, "D" Type, Rivers.

Rabbit No.	Material injected.	No. of bacteria injected.	Duration of life in days.
1	1 cc. blood broth culture.	600,000,000	1
2	1 " pleural exudate: Rabbit 1.	1,700,000,000	3
3	1 " " " " 2.	1,000±	2
4	1 " " " " 3.	9,200,000,000	1
5	1 " " " " 4.	85,000,000,000	1
6	1 " " " " 5.	120,000,000,000	2
7	1 " " " " 6.	30,000,000,000	1
8	1 " " " " 7.	200,000,000	1



TEXT-FIG. 2. Virulence of 9 passage culture and "unpassed" stock culture of *Bact. lepisepticum*. Rivers "D" strain.

"Control (II)" represents preliminary titration (Table II).

and 1:10,000,000. 6 rabbits were injected intranasally with the undiluted culture. At the same time, an 18 hour rabbit blood broth culture from the unpassed stock, Rivers strain, was injected intrapleurally as follows: 1:10, and 1:100, 1 rabbit each; 1:10,000, and 1:1,000,000, 2 rabbits each. 6 rabbits were also injected intranasally with this culture. The number of organisms per cc. in the original cultures was determined as usual.

Of the 6 rabbits receiving the passed culture intranasally, 1 died with pleuropneumonia and the other 5 developed snuffles. Of the 6 rabbits receiving the unpassed culture intranasally, 2 died with pleuropneumonia and 2 more developed snuffles.

The results of the intrapleural inoculation are summarized in Text-fig. 2. The chart includes the results of the preliminary control titration, as well as those of the passed and unpassed cultures, and shows that there was no significant difference in duration of life between animals receiving the "passed" and those given the "unpassed" cultures. Hence, it is concluded that animal passage, as described in this experiment, does not affect the virulence of the "D" Rivers strain.

Experiment 3.—A culture of *Bact. lepisepticum*, Type "G," was obtained from the nares of a rabbit at the onset of spontaneous snuffles, and designated as Strain "R. 82." It was grown for 16 hours in blood broth and then injected intrapleurally

TABLE X.

Direct Pleural Passage of Bact. lepisepticum, "G" Strain, "R. 82."

Rabbit No.	Material injected.	No. of bacteria injected.	Duration of life.
1	1 cc. blood broth culture.	470,000,000	7 days.
2	1 " pleural exudate: Rabbit 1.	200,000,000	5 "
3	1 " " " " 2.	5,000,000,000	1 day.
4	½ " heart's blood: " 3.	?	3 days.
5	1 " pleural exudate: " 4.	?	1 day.
6	1 " " " " 5.	?	12 days.
7	Culture from abscess in chest wall of Rabbit 6.	?	2 "
8	1 cc. pleural exudate: Rabbit 7.	?	6 hrs.
9	1 " bloody pleural fluid: Rabbit 8.	?	4 days.
10	1 " pleural exudate: Rabbit 9.	?	2 "

into six 1000 gm. rabbits in the following dilutions: 1:1, 1:10, 1:1000, and 1:100,000. The actual number of bacteria injected was determined by the plating method. The animal receiving 470,000,000 bacteria lived 7 days; the one receiving 47,000,000 survived; the 2 given 470,000 died in 5 days; and the 2 given 4700 survived.

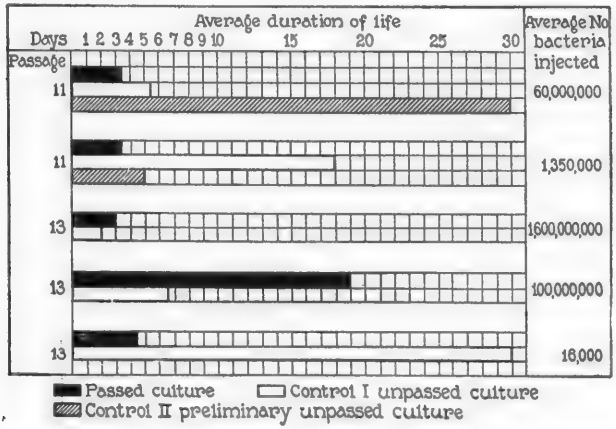
From the first rabbit of this series to die, 1 cc. of pleural exudate was taken and injected intrapleurally into another rabbit of similar weight. A bacterial count was also made. This procedure was then repeated through a series of 10 rabbits as shown in Table X. Probably over 1 billion bacteria were injected in every case. The survival times varied from 6 hours to 12 days.

From the tenth passage rabbit, 1 cc. of pleural exudate was taken and injected intrapleurally into 6 rabbits in the following dilutions: 1:20, 1:200, and 1:20,000. At the same time, a 16 hour rabbit blood culture from the unpassed strain was injected into 6 similar rabbits in the following dilutions: 1:2, 1:20, and 1:2000.

A summary of the results, plotted in Text-fig. 3, shows that in the two instances in which the numbers of bacteria injected were at all comparable, the animals receiving the "passed" culture died sooner than those of the "unpassed" series.

This apparent change in virulence was tested further by a second titration. The "passed" culture and pleural fluid from the first rabbit to die in the above titration series were injected directly into the thoracic cavity of another animal. After one more direct passage, the culture from the pleural fluid was inoculated into 6 rabbits in dilutions of 1:10, 1:100, 1:10,000, and 1:1,000,000, and the unpassed control culture was inoculated in dilutions of 1:1, 1:10, 1:1000, and 1:100,000.

The results of this titration are also given in Text-fig. 3, and in this case show that animals given the passed culture lived somewhat longer than the controls



TEXT-FIG. 3. Virulence of 11 and 13 passage culture and "unpassed" stock culture of *Bact. lepisepticum.* "G" strain, R. 82.

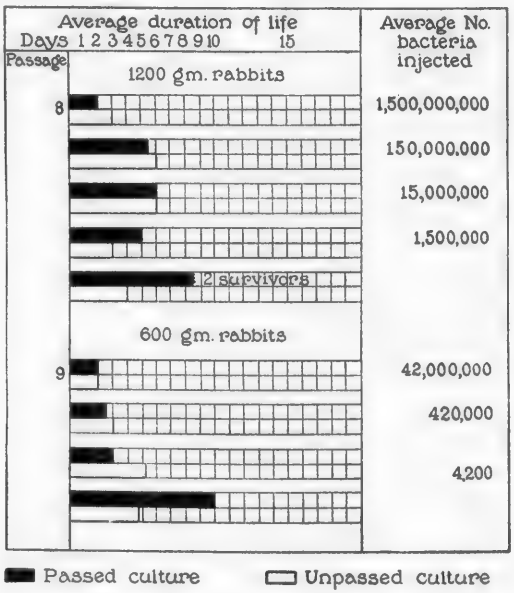
given quantitatively similar doses of the unpassed culture. One must conclude, therefore, when the results of the entire experiment are considered, that animal passage did not induce a significant change in the virulence of Culture "G" No. 82.

Experiment 4.—A mucoid strain, No. 329, obtained from a case of spontaneous snuffles, was passed in the same manner. 800 gm. rabbits from the breeding room were used and the material was introduced intrapleurally in ½ cc. amounts. Each rabbit dying of the infection was autopsied with care, and cultured to see if any type changes were occurring.

The first animal received ½ cc. of an 18 hour blood broth culture, and died on the following day. ½ cc. of its pleural exudate was then given to 2 more rabbits. They died on the 1st and 2nd days following. Pleural fluid from the first one to die was given to 2 more; they died on the 2nd day. Those receiving the fourth passage

material died on the 1st and 4th days; the next 2 on the 1st and 2nd; the next on the 1st and 2nd, and the seventh passage couple on the 1st and 2nd days. Plates from all these animals showed pure cultures of typical mucoid colonies.

Pleural fluid from the first seventh passage rabbit to die was diluted in salt and given intrapleurally to 10 rabbits. An additional 10 rabbits were injected in a similar manner with the unpassed stock culture. Finally, from 1 of the rabbits receiving this seventh passage culture, pleural fluid was again diluted and given to 8 more animals. 8 controls were given the unpassed culture at the same time. Counts were made and the duration of life of the rabbits receiving the passed cul-



TEXT-FIG. 4. Virulence of 8 and 9 passage culture and "unpassed" stock culture of *Bact. lepisepticum*. Mucoid strain, 329.

ture was compared to that of the animals which had been given a similar number of the unpassed bacteria.

The results of these titrations are plotted in Text-fig. 4, and show that no significant difference occurred in the survival time of animals receiving the "passed" and "unpassed" cultures.

We conclude, therefore, that this method of rapid animal passage does not change the virulence or type characteristics of the mucoid *Bact. lepisepticum*.

The above experiments indicate that passage of *Bact. lepisepticum* through rabbits, its native host, by way of an abnormal portal of entry, does not affect its virulence. In the following tests, animal passage was effected by way of the normal portal of entry, that is, the nares and upper respiratory tract.

Experiment 5.—Rabbits used in this experiment came from the Rockefeller Institute breeding room, the entire population of which has been free of snuffles and *Bact. lepisepticum* carriers for 2 years. The "D" culture, "Rivers," was employed.

Six 800 gm. rabbits were given ¼ cc. of an 18 hour rabbit blood broth culture into each external naris by means of a blunt syringe. The fluid was introduced without touching the mucous membranes.

3 days later, 1 rabbit died of pleuropneumonia. An 18 hour heart's blood broth culture was given in the same manner to 2 more rabbits. 1 of these showed signs of snuffles after 24 hours; the other died 9 days later of pleuropneumonia. A blood broth culture from this rabbit was given intranasally to 3 more rabbits. 2 of them developed snuffles and 1 died after 9 days with pleuropneumonia. From this animal, a heart's blood broth culture was taken and after 17 hours' incubation was given in similar doses of ¼ cc. to 3 more animals. These 3 rabbits remained clinically well. Similar results were obtained with other "D," mucoid, and "G" strains.

It is concluded, therefore, that under the conditions of these experiments, the passage of *Bact. lepisepticum* through a series of hosts of the native species, by way of the normal portal of entry, does not increase the virulence of the organism.

DISCUSSION AND SUMMARY.

As a preface to a general discussion of these experiments, we wish to refer again briefly to the titrations of virulence described in this paper. None of them was carried out in an altogether proper manner,—it was necessary to employ rabbits of mixed breed, to use small numbers, and in some instances, to compare the results of titrations done at different times of the year. Furthermore, many tests were made in the mouse, a foreign host, and by means of artificial injection into the peritoneal cavity. Hence, if our results had shown fluctuations and wide differences, we should have experienced difficulty in interpreting their significance.

Fortunately, however, the control measures which we were able to employ,—namely the utilization of animals of similar age and weight,

bred at the Rockefeller Institute under uniform environmental conditions, and free of previous exposure to *Bact. lepisepticum*, together with the technique of intranasal instillation of similar doses,—were sufficient to give consistent and relatively uniform effects. These we have regarded as presumptive evidence of the following: (1) that of the three types of *Bact. lepisepticum* described, "D" strains are the most virulent or pathogenic, mucoid forms less so, and "G" types little or not at all virulent; (2) that different strains of the same type are of similar virulence; and (3) that passage of type-pure strains through non-toxic, nutrient media, or through animals, does not modify their virulence.

The experiments described in the present communications were planned to investigate certain microbic factors which seem to influence the spread of *Bact. lepisepticum* infection. Accordingly, we studied the types encountered in nature, their behavior in the rabbit host, and other qualities which might possibly be related to their parasitic activities.

At the outset we discovered that very little is known about the "Pasteurella" or "hemorrhagic septicemia" organisms. Every small, blunt bacillus with bipolar granules and certain quite definite fermentative reactions is placed in this group and is further designated by prefixing the name of the animal from which it was obtained. Differentiation on the basis of serological studies has been extremely difficult and as yet not sufficiently detailed and complete to be of use. We have, therefore, called our strains "*Bact. lepisepticum*," because they belong to the Pasteurella group and were obtained from rabbits, but at the same time have recognized the possibility that similar forms may also be present in other animal hosts.

The "D" and mucoid types may or may not be mutually related. Each has its distinctive growth characteristics and antigenic properties, but these differences are not sufficient to exclude the possibility that the two types may have resulted from the action of bacteriophage or so called "mutation" phenomena upon a single "parent" strain.

We have attempted to explain the difference in natural prevalence of "D" and mucoid types in terms of virulence and vegetative capacity (1, *a*, *d*). Thus we find that "D" types are at present rarely encountered in rabbits, while the mucoid forms are widespread. The

former prove to be highly virulent and slightly vegetative; the latter, less virulent and readily vegetative. The same inverse relationship between virulence and prevalence obtains in human diphtheria (7) and pneumococcus infections (8);—virulent Type I and II pneumococci and diphtheria bacteria are relatively uncommon in carriers, while the less pathogenic diphtheria forms and Type III[2] and IV pneumococci are widespread. It is possible, therefore, that in these respiratory diseases the virulent types of bacteria are transients, unable to survive in a community except at epidemic times, while the less pathogenic types are more adapted to a parasitic existence in the tissues of a considerable percentage of a population (9).

Just what properties of bacteria are responsible for their ability to kill or to survive indefinitely in the surface tissue of an animal has not been determined. We find that physical properties such as agglutinability in acid buffer solutions, stability in suspension, and potential, bear no consistent relationship to virulence and vegetative capacity. Qualitative fermentation and indole reactions, likewise, gave no significant results. Experiments relating to comparative growth rates and nutritional requirements have, however, shown the following differences: Virulent "D" types were found to require a medium with relatively low oxygen tension and these multiplied with extreme rapidity; mucoid types were less exacting and grew more slowly; while "G" variants needed no accessory substances for good growth. Hence, there may prove to be some relation between food supply, metabolic activity, and pathogenicity; bacteria may be parasitic or saprophytic because of their own specific nutritional requirements, and may be virulent in one host and not in another because of chemical differences in the available food. It will be interesting to test the validity of these suggestions by further experiment.

We may summarize the results of this investigation as follows. A "D" and a "mucoid" type of Pasteurella organisms have been recovered from rabbits; the former is relatively rare, is virulent, and not vegetative; the latter is very common, less virulent, and readily

[2] We consider Types III and IV to be less virulent than Types I and II because of the relatively low ratio of mortality per cent to incidence per cent. We also note a very interesting experimental study by M. J. Rosenau and his associates which tends to confirm this belief (*Am. J. Hyg.*, 1926, vi, 463).

adaptable to a vegetative existence. The virulence of the different strains of each type appears to be about the same and not to be affected by passage through animals or non-toxic, nutrient media. Although each possesses distinctive and constant characteristics, we feel that the possibility of mutual relationship has not been entirely excluded. Investigation of bacterial properties associated with virulence has as yet given no definite and positive results.

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ACTION ON FIBROBLASTS OF THE PROTEIN FRACTION OF EMBRYONIC TISSUE EXTRACT.

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It is known that various tissues will grow indefinitely *in vitro* in a medium consisting of one part of plasma and one part of embryonic tissue juice,[1] and that it is the embryonic tissue juice and not the plasma that furnishes the necessary substances for cell nutrition and multiplication.[2] Whether there is in embryonic extract a specific substance with a function of initiating or producing cell division, or whether the embryonic extract simply contains the essential nutrient substances required by the cells, is not known. Neither is there any knowledge concerning the chemical nature of the substances present in the extract which are utilized by the cells. The work reported in this paper is a preliminary examination of the action of the protein-containing fraction of embryonic extract in relation to its ability to promote the growth of fibroblasts in pure culture, or from heart tissue.

The protein of the embryonic tissue extract was precipitated in a variety of ways and redissolved in a volume of Tyrode solution equal to the volume of tissue juice from which the precipitate was obtained. It was found that the protein precipitate, provided it could be entirely freed from the reagent used for its precipitation and again brought into solution, contained some of the growth-stimulating action of the original extract. Ammonium salts, trichloracetic acid, picric acid, pyridine, etc., were too difficult to remove and were toxic to the tissues. Carbon dioxide passed through a diluted solution, ethyl,

[1] Carrel, A., *J. Exp. Med.*, 1912, xv, 516; 1913, xvii, 14; 1914, xx, 1. Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367. Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755. Carrel, A., and Ebeling A. H., *J. Exp. Med.*, 1923, xxxviii, 487. Ebeling, A. H., *J. Exp. Med.*, 1925, xli, 337.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317. Carrel, A., *J. Am. Med. Assn.*, 1924, lxxxii, 255; *Brit. Med. J.*, 1924, ii, 140.

methyl, and isopropyl alcohol, acetone, and acetic acid, all yielded precipitates which showed decided activity. When a comparison was made between these precipitates and the original extract, none was found to be quite as active as the original. However, this was not surprising for, if a sufficient concentration of precipitant such as acetic acid or alcohol was used, some of the protein became denatured as could be seen by a difference in the translucency of its solution. When a sufficiently small concentration of the precipitant was used to prevent denaturation, a part of the protein was not precipitated and the filtrate as well as the precipitate possessed growth-promoting properties. To afford some basis of comparison the method was adopted, therefore, of determining the concentration of nitrogen in the solution of the precipitate or the filtrate, and in the original extract, and then diluting the original extract with Tyrode solution so that it contained the same per cent of nitrogen as the experimental medium.

Tests on a number of precipitates were made in which the cultures were kept for only a few passages. In most cases, the tissue cultivated in the control extract and that in the solution of the protein precipitate grew at the same rate. No case occurred in which the precipitate showed any greater activity than the original extract. In some cases it was less active, because the method of fractionation either denatured the protein or destroyed some activating substance, for the activity of embryo juice is easily destroyed by heat, aging, and many chemical substances. Precipitation by alcohol, even at low temperature, always gave a precipitate less active than the original extract.

A fractionation of the protein by alcohol was carried out by adding a small amount of alcohol, centrifuging the precipitate, and then increasing the concentration of alcohol, etc., until ten fractions were obtained. The first three or four of these precipitates were quite active, the activity decreasing as the concentration of alcohol increased until practically no activity was obtained from the last fractions. The filtrate from precipitation by an equal volume of alcohol contains a considerable amount of active substance, also a considerable quantity of protein. A 50 per cent alcoholic solution will extract from embryo

pulp an appreciable quantity of protein and produce an active extract when the alcohol is removed.

A few adsorption experiments were also tried, with charcoal, kaolin, and alumina as adsorbents. If a sufficient quantity was used to adsorb all the protein, the filtrate remaining was without any noticeable activity. If all the protein was not adsorbed, the filtrate generally exhibited some activity. The experiments tried on redissolving the active substances from the adsorbents were not successful.

These preliminary tests indicate that the protein of the extract is the essential nutritive substance upon which fibroblasts live *in vitro* and if, besides the protein, there is some specific substance essential for cell multiplication, it is united to the protein by a chemical or physical bond or is readily adsorbed on the protein as it precipitates.

Experiments on cultures in which the tissue is grown for only one passage of 2 or 3 days are valuable for preliminary information but are not conclusive, since it is quite probable that the tissue may carry along with it from its old medium, either mechanically or already adsorbed, some substance essential for its growth. The only convincing experiments, therefore, are those in which the tissues live for several passages in the experimental medium and its control. This is a time-consuming process, so as yet only results with the protein precipitated by carbon dioxide and its filtrate have been procured in this way. Tests have been made on the precipitate, using the method of small cultures in which the tissue is cut at every passage,[3] and also that of cultivation in flasks[4] where the tissue is not removed but the total accumulated growth over the entire period may be observed (Figs. 1 to 3). It is evident from the curves that the rate of growth of the tissue in the protein precipitated by CO_2 and redissolved in Tyrode is approximately the same as that in the original extract diluted to contain the same per cent of protein. The condition of the cells in the experimental medium was as good throughout as in the control. Since the experiment (Fig. 1) was carried on for 28 days, it seems conclusive that the protein precipitate contains all the necessary nutritive substances. Only a fraction of the protein, however, is precipi-

[3] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.
[4] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

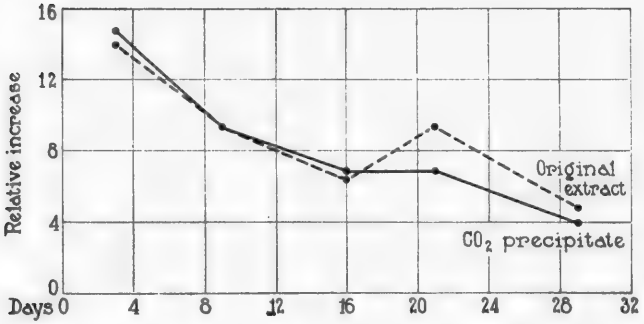

FIG. 1. Comparison of the rate of growth of fibroblasts in the protein precipitated by CO_2 and in the original extract diluted to the same nitrogen content.

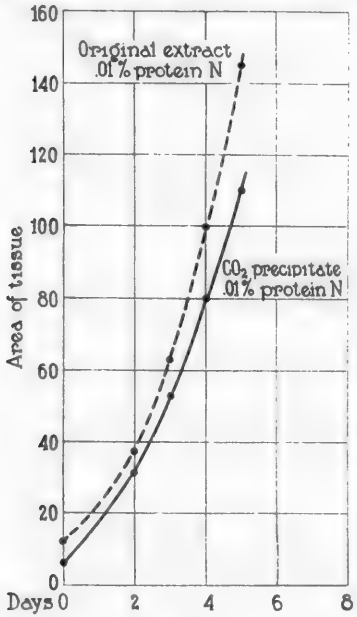

FIG. 2.

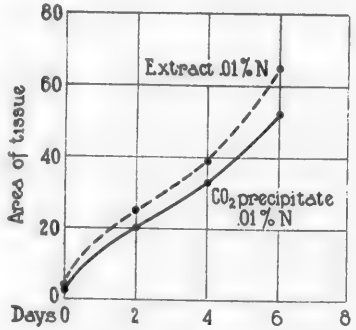

FIG. 3.

FIG. 2. Comparison of the rate of growth of fibroblasts from embryo heart in embryo extract and in the protein precipitated from it by CO_2.

FIG. 3. Comparison of the rate of growth of a 13 year old strain of fibroblasts in embryo extract and in the protein precipitated by CO_2.

tated by CO_2, approximately one-half of that originally present. The filtrate is also quite active (Fig. 4), and relatively in proportion to its protein content. A careful examination of the curves for the CO_2 precipitate and the CO_2 filtrate shows that there is a little activity due to the non-protein nitrogen present. This causes a slightly increased

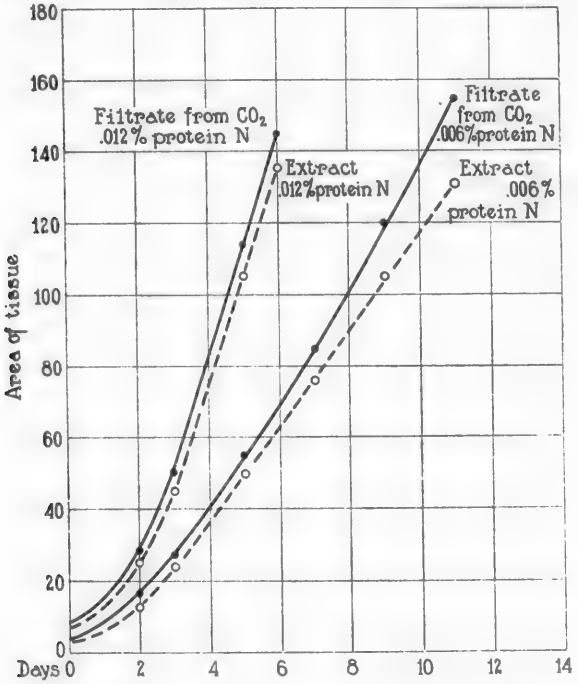

FIG. 4. Comparison of the rate of growth of fibroblasts from embryo heart in embryo extract and in the CO_2 filtrate containing equal concentrations of protein nitrogen.

area in the growth of tissue in the extract over that in the CO_2 precipitate, which contains only protein nitrogen and a slightly greater area in the CO_2 filtrate than in the diluted extract. The basis of comparison here was an equal content of protein nitrogen. As has been shown previously[5] amino acids are capable of producing a slight

[5] Carrel, A., and Ebeling, A. H., *Compt. rend. Soc. biol.*, 1924, xc, 31.

stimulation of growth, causing greater migration of the cells, but they do not produce an increase in the mass of the tissue, as is done by the protein fraction of embryo juice. Further confirmation of these findings is reported in the following paper.

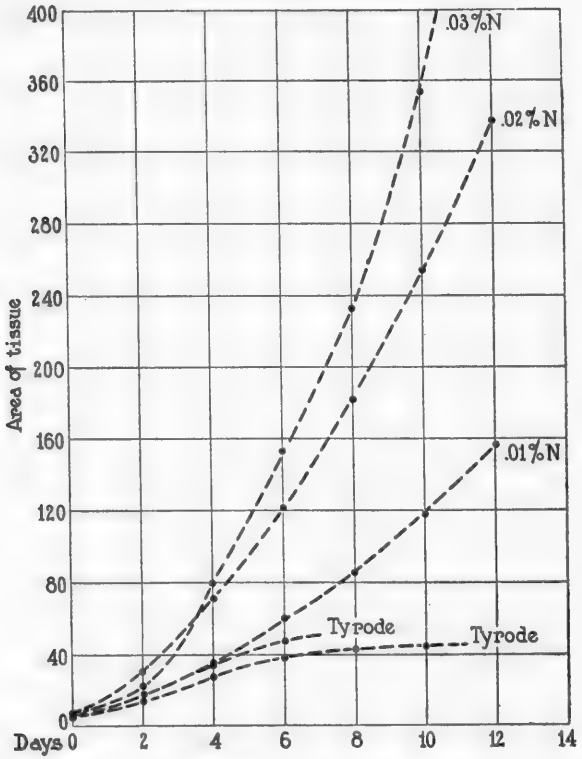

FIG. 5. Rate of growth of fibroblasts in embryo extract diluted to contain 0.01, 0.02, and 0.03 per cent of nitrogen. Note that the shape of the curve for media containing nutrient materials is quite different from that for Tyrode solution.

The rate of growth of fibroblasts is proportional to the concentration of active substances in the medium, a fact well illustrated by their growth in embryonic tissue extract at different dilutions. In the experiments recorded in Fig. 5, one sample of tissue juice was diluted so as to contain 0.01, 0.02, and 0.03 per cent nitrogen. Tissues do not,

however, grow at the same rate in different preparations of extracts, even when they contain the same per cent of protein. Curves such as those figured are, therefore, truly indicative of the concentration of growth-activating substances in the experimental medium when compared with a sample of the original extract from which the experimental medium was prepared.

Since the protein of the embryonic tissue extract appears to contain the essential nutritive and activating substances, experiments were undertaken to purify it by the process of repeated precipitation. This

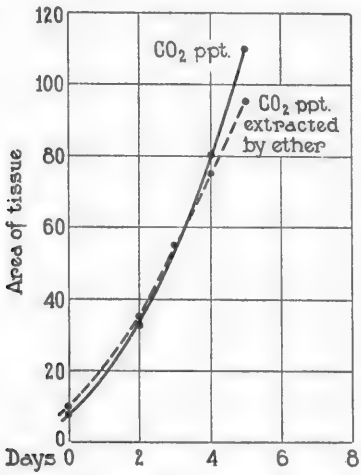

FIG. 6. Comparison of the rate of growth of tissue in the CO_2 precipitate and the CO_2 precipitate extracted by ether.

was tried with the precipitates obtained by carbon dioxide, acetic acid, and alcohol. The precipitation was carried out at low temperature and, if only a part of the total protein was precipitated, it was dissolved in a correspondingly smaller volume of Tyrode solution before being reprecipitated. The activity of the first and third precipitates was tested. The third precipitate was found to be considerably less active in each case; in fact, when it was compared with Tyrode solution, it was found to be almost inactive. Obviously, the protein has either lost some substance attached to it which was essential to the growth of the tissues or it has itself been altered in the process of

purification so as not to be biologically the same, even if it appears to have the same chemical properties.

The protein of embryonic tissue juice has been tested chemically and found to be a mixture of nucleoprotein and a glycoprotein with mucin-like properties. However, when these proteins were isolated in a comparatively pure state from embryo pulp and tested on growing tissues, they did not seem to possess any growth-promoting action.

Pure sodium nucleate from embryonic pulp was prepared and found to be inactive, as were many other substances also, among which were egg albumin, egg globulin, crystalline egg albumin, nucleoalbumin, nucleoglobulin, lecitho-albumin, thymus nucleic acid, and acid metaprotein from crystalline egg albumin. Crystalline egg albumin prepared with ammonium sulfate proved to be toxic even after prolonged dialysis, but that from which the ammonium sulfate was removed by recrystallizing three times with Na and K sulfates, and washing with sodium chloride and acetic acid, was not toxic. A few of the above preparations appeared to be slightly stimulating in the first passage but proved inhibiting after three passages, and in no case was there any growth as large as that obtained with even a very small quantity of embryo tissue extract.

Since the protein precipitated by almost any method carries along with it by adsorption some substances of lipoid nature, these precipitates and the original extract were purified by extraction with ether. As will be reported in another paper of this series, the growth-promoting action was not lost (Fig. 6). Therefore, it is the protein, and not the lipoid associated with it, that carries the activity of the embryo tissue extract.

DISCUSSION AND SUMMARY.

The above experiments indicate that the growth-stimulating substance found in embryonic tissue extract, which has been responsible for the continuous growth of fibroblasts *in vitro* for 14 years, is either protein in nature or closely associated with the protein of the extract and adsorbed by it. If any specific hormone responsible for cell division is present, it is united to the protein or carried along with it in its first precipitation. It seems probable that the tissues utilize this protein for the nitrogen which they build into protoplasm. Whether it

is first hydrolyzed before adsorption by the tissues has not been ascertained as yet. It has been shown in other experiments reported in the following paper that the amino acids of the tissue juice do not suffice for the growth of fibroblasts and that hydrolyzed tissue juice is toxic in the same way that a too concentrated mixture of amino acids is toxic.

The results of the foregoing experiments may be summarized as follows:

1. Fractionation of embryo tissue juice has shown that it is the protein fraction that contains the activating substance.

2. Tissues continue to grow for a long time in the protein of the extract precipitated by CO_2 and at a rate approximately equal to that in the original extract diluted to the same nitrogen concentration.

3. The non-protein nitrogen gives slight stimulation to growth.

4. Purification of the protein by repeated precipitation destroys its growth-promoting properties, but whether this is due to a denaturing of the protein,—which occurs very readily,—or to loss of some substance possibly an enzyme attached to it, has not been ascertained.

5. Preparations of purified proteins from embryonic tissue and egg white have shown no marked nutritive or stimulating action. A number of other pure substances have been tried without effect.

The authors wish to express their thanks to Dr. Michael Heidelberger for his criticisms and suggestions in connection with this work.

EFFECT OF THE AMINO ACIDS AND DIALYZABLE CONSTITUENTS OF EMBRYONIC TISSUE JUICE ON THE GROWTH OF FIBROBLASTS.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 15, 1926.)

It is known that embryonic tissue juice is essential to the continuous growth of connective and epithelial tissues *in vitro*;[1] but the nature of the chemical constituent or constituents present in the proteins of the embryonic tissue extract,[2] which give to it its peculiar growth-promoting properties, has not been discovered. This paper will deal with the separation of the extract into its dialyzable and non-dialyzable constituents and the action of each on the growth of fibroblasts *in vitro* with special reference to the amino acid content of the two fractions. The amino acids were separated from the rest of the extract in order that evidence might be obtained as to whether the nitrogen required for the synthesis of protoplasm is utilized in the form of amino acids.

Since the proteins upon which animals subsist are broken down into amino acids before entering the blood stream and since animals may be maintained in nitrogen equilibrium on enzyme digests of proteins consisting almost entirely of amino acids, it would seem quite probable that the tissues utilize nitrogen in this form. It has been found that the amino acids of serum are incapable of supporting the growth of fibroblasts in pure culture,[3] but whether or not this is true *in vivo*, where the circulating blood brings constantly renewed supplies, has

[1] Carrel, A., *J. Exp. Med.*, 1912, xv, 516; 1913, xvii, 14; 1914, xx, 1. Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367. Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 487. Ebeling, A. H., *J. Exp. Med.*, 1925, xli, 337.

[2] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xliv, 387.

[3] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxvii, 759.

not been ascertained by the experiments thus far conducted *in vitro*. Extensive experiments carried out in this laboratory with many individual amino acids and also with mixtures have shown that the acids stimulate the tissues and produce a greater area of migration, but that they do not prolong the life of the tissues and in themselves are not sufficient to bring about continued multiplication of cells.[4] On the other hand, embryonic tissue juice may contain certain specific acids essential to the growth of the tissues which are lacking in serum and in artificial mixtures of amino acids.

The study of the effects of the amino acids and dialyzable constituents of embryonic tissue juice was carried out in several ways.

1. The embryonic tissue extract was dialyzed in very permeable collodion sacks until free from amino acids, and compared with the original extract for its growth-promoting properties.

2. The dialyzed extract was tested to ascertain whether an enzyme were present which produced an increase in the amino acids after those originally present were removed.

3. The amino acids were separated from the extract by ultrafiltration along with other ultrafilterable constituents, and their action was tested for their growth-promoting effect.

4. An artificial mixture of sixteen amino acids was added to extract dialyzed until free from amino acids, and this was compared with the dialyzed extract.

5. The ultrafiltrate was added to extract from which the acids had been removed by dialysis and this was compared with the extract free from amino acids.

6. The amino acids obtained by hydrolyzing embryonic tissue juice, both by acid and by trypsin, were tested at various concentrations for growth-promoting action.

The technique of Carrel and Ebeling[5] for the cultivation of the tissues was used, the two halves of the same culture being carried through several passages, one in the experimental medium and one in the control medium. Measurements of the area of growth were made every 2 days.

The Action of Embryonic Extract, Dialyzed until Free from Amino Acids, on the Growth of Fibroblasts.

In order to remove the amino acids completely as rapidly as possible from the extract, collodion bags of high permeability were used.

[4] Carrel, A., and Ebeling, A. H., *Compt. rend. Soc. biol.*, 1924, xc, 31.

[5] Carrel, A., *Compt. rend. Soc. biol.*, 1923, lxxxix, 1017. Ebeling, A. H., *J. Exp. Med.*, 1921, xxxix, 231.

The bags were prepared according to the technique of Gates,[6] on gelatin capsules to which a glass neck had been sealed. The capsules were dipped once in a thick collodion solution, drained, and exposed to the air for 2 minutes, then immersed in 95 per cent alcohol for 10 minutes, and hardened in cold water. After the gelatin was washed out with hot water, they were preserved in 95 per cent alcohol until desired for use, at which time the alcohol was washed out with sterile water and the bag itself subjected to dialysis until all traces of alcohol were removed. Tissue extract dialyzed in these sacks became free from amino acids in 20 to 30 hours. Determinations of amino nitrogen were made from time to time in the Van Slyke micro apparatus, until a minimum amount of amino nitrogen remained, *i.e.*, only the quantity characteristic of the protein itself as separated from the extract by precipitation methods. In a number of the experiments, running water was circulated in a narrow coil immersed in ice before coming into contact with the dialysis membrane. In others, tap water at 12–15°C. was used.

The dialyzed extracts were made isotonic either by the addition of an equal volume of double strength Tyrode solution or by evaporating to dryness and redissolving in ordinary Tyrode solution. In either case, the concentration of protein nitrogen was determined in the dialyzed extract and in a sample of the original extract saved as a control, and dilutions were made to bring the two to the same per cent of protein. The pH was also adjusted to the same value, approximately 7.6.

All the samples of dialyzed extract, in spite of differences in details of preparation, gave the same result when tested on growing tissues. The growth stimulating substance was not entirely removed, for the rate of growth was considerably greater in the dialyzed extract than in Tyrode solution (Fig. 1). A very much smaller area of growth was obtained in the dialyzed extract than in the original one (Figs. 2 and 3). This difference, however, may be attributed to two causes: the loss of the amino acids and other dialyzable constituents, or to a denaturation of part of the protein through the long continued action of water. The dialyzed extract often exhibited a greater opalescence than the original one, especially in reflected light, indicating a change in the physicochemical state of the protein. Such preparations gave very poor growth. Others showed a much smaller decrease in growth-promoting properties, both the experiment and control remaining in good condition.

Since it is conceivable that the extract dialyzed until free from

[6] Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 25.

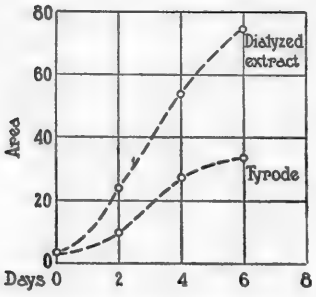

FIG. 1. Rate of growth of fibroblasts from embryo heart in embryo tissue juice dialyzed free from amino acids. Compared with growth in Tyrode solution. Cultures in flasks.

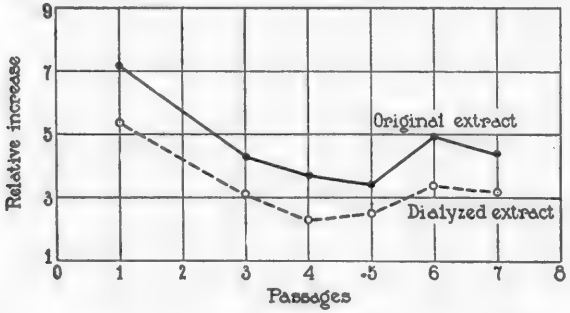

FIG. 2. Comparison of the growth of an old strain of fibroblasts in embryo extract and in the same extract dialyzed free from amino acids. Concentration of nitrogen is the same in each case.

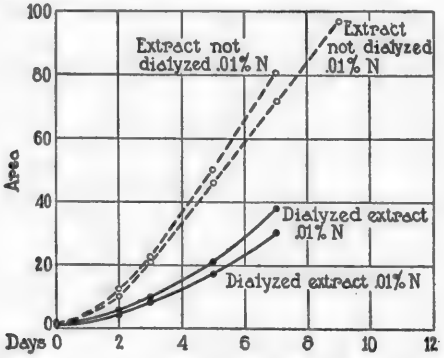

FIG. 3. Comparison of the rate of growth of fibroblasts in embryo tissue extract and in extract dialyzed free from amino acids. Cultures in flasks.

amino acids might not remain so, owing to the action of enzymes on its protein, the dialyzed extract was tested at various pH values for a regeneration of its amino acids. The increase of amino nitrogen was so slow that growth of tissues in this dialyzed extract could not be attributed to that cause. Therefore, it appears that the nitrogenous substances utilized by the fibroblasts growing in dialyzed extract are other than amino acids.

The Action of the Amino Acid Fraction of Tissue Juice Obtained by Ultrafiltration.

It was found that the amino acids of the tissue extract could be separated from the protein by a process of ultrafiltration under pres-

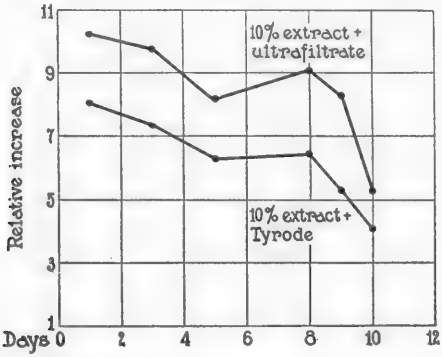

FIG. 4. Effect of the ultrafilterable constituents of embryo tissue juice on the rate of growth of fibroblasts.

sure through a collodion sack prepared exactly as those used for the dialysis. No protein passed through these sacks, *i.e.*, the ultrafiltrate gave none of the tests for protein, and there was no increase in amino nitrogen after hydrolysis by acid. The amino nitrogen content of the ultrafiltrate was found to be almost equal to that of the original tissue juice, the difference being due to amino groups attached to the protein molecule. Experiments on growing fibroblasts, with this ultrafiltrate, demonstrated that the tissues lived no longer in it than in Tyrode solution, although the area of growth at each passage was

slightly larger (Table I). The behavior was quite analogous to that of tissues in artificial mixtures of amino acids reported by Carrel and Ebeling.[4] When 10 per cent of fresh embryonic extract was added to the ultrafiltrate and also to the Tyrode solution, in order to prolong the life of the tissues, it was found that a larger area of growth was obtained in the presence of the ultrafiltrate at every passage (Fig. 4), although the difference was not sufficient to increase the mass of the tissues in one case more than in the other. It is, therefore, only an increase in the area of migration and not in the mass of tissue that

TABLE I.

Rate of Growth of Fibroblasts in the Ultrafiltrate of Embryo Juice. Tyrode Solution Used as Control.

Group No.*	Preparation No.	Rate of growth in		Ratio: *E/C.*	Remarks.
		Tyrode (*C*).	Ultrafiltrate (*E*).		
6304	X-3	5.93	8.02	1.35	1st passage.
6312	X-3	5.85	7.90	1.35	2nd "
6327	X-3	5.57	8.20	1.47	3rd "
6276	X-3	4.03	5.85	1.45	1st "
6314	X-6	8.8	9.6	1.09	1st "
6328	X-6	5.27	7.76	1.47	2nd "
33268	X-26	4.88	5.2	1.06	1st "
33286	X-26	5.0	5.0	1.0	2nd "
33297	X-26	4.71	5.95	1.26	3rd "
Average.....				1.28	

* Each group consists of three or four experiments.

is produced by the amino acids and other ultrafilterable constituents of embryo tissue juice.

The residue from the ultrafiltrate retained some growth-stimulating action, but so much of the protein became insoluble in the process that it was not a valuable experimental material.

Effect of Adding the Ultrafiltrate or an Artificial Mixture of Amino Acids to Dialyzed Extract.

Since the removal of amino acids from tissue extract by dialysis diminishes its growth-promoting action, an experiment was tried to

see whether the addition of the amino acids and other dialyzable constituents obtained by ultrafiltration would restore this action. Fig. 5 illustrates the rate of growth of tissues in the dialyzed extract,

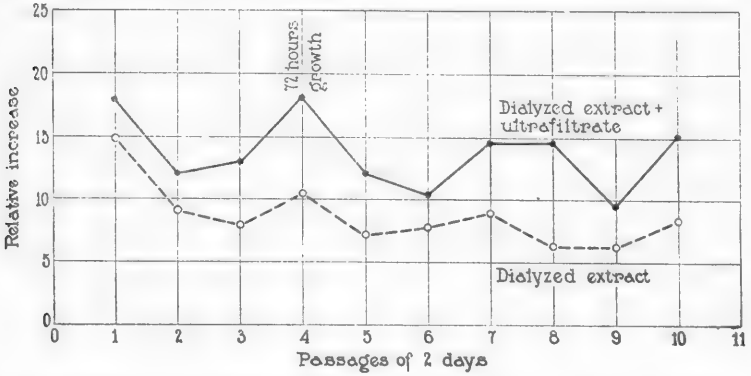

FIG. 5. Effect of replacing the constituents removed by dialysis with the ultrafiltrate of embryo tissue juice on the rate of growth of fibroblasts.

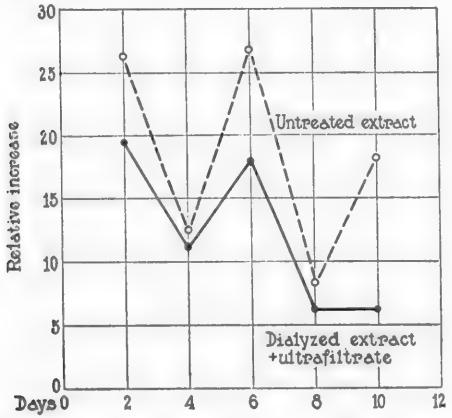

FIG. 6. Comparison of the rate of growth of fibroblasts in dialyzed extract plus the ultrafiltrate with that in the original extract.

and in the dialyzed extract to which the ultrafiltrate has been added. It appears that some of the lost activity is restored, for there is a slight increase in the migration of the cells. It is not all restored however,

for when this same dialyzed extract containing the ultrafiltrate was compared with a sample of the original extract not treated in any way, the growth in the original extract was somewhat better (Fig. 6). This phenomenon is probably not due to any difference in the amino acids of the embryonic extract, but to a denaturing of part of the protein by the action of water, and by changes in temperature and concentration.

Finally, in order to ascertain whether the amino acids or some other ultrafilterable and dialyzable constituents were responsible for the effect observed, experiments were made in which an artificial mixture of sixteen amino acids was added to the dialyzed extract. The results

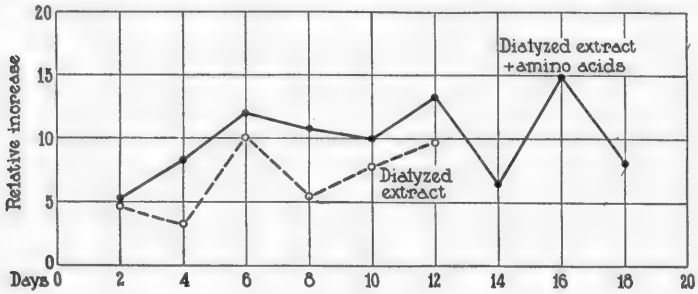

FIG. 7. Effect of the addition of an artificial mixture of amino acids to dialyzed embryo extract on the rate of growth of fibroblasts.

were quite analogous to those obtained on adding the ultrafiltrate (Fig. 7). In all cases, the presence of the amino acids or ultrafiltrate gave a larger area of migration at each passage, but this did not bring about any increase in the mass of the tissue.

Action of Amino Acids Obtained by Hydrolyzing Embryonic Tissue Juice by Acid and by Trypsin.

Since the concentration of amino acids in the embryonic extract and in the ultrafiltrate is quite small (6 to 12 mg. per 100 cc.), it was thought that a higher concentration of the acids obtained by the hydrolysis of the tissue juice protein with acid or trypsin might produce decided growth, although those already present had failed to do so. These digests, however, proved toxic even when diluted to such an

extent that they contained very little more amino nitrogen than an ordinary embryonic extract (Table II).

TABLE II.

Rate of Growth of Fibroblasts in Amino Acids Obtained by Hydrolysis of Embryo Juice Protein.

Tyrode Solution Used as Control.

Group No.*	Preparation No.	Rate of growth in		Ratio: *E/C.*	Remarks.	
		Tyrode (*C*).	Ultrafiltrate (*E*).		Passage.	
34–144 C	X-69	9.1	7.5	0.82	1st	Hydrolyzed by acid.
9 C	X-69	12.0	9.7	0.81	2nd	Tryptophane and
20 C	X-69	10.7	8.0	0.75	3rd	cysteine added.
34 C	X-69	2.8	1.0	0.36	4th	
8 C	X-70	11.1	7.2	0.65	1st	
21 C	X-70	8.45	4.71	0.56	2nd	Hydrolyzed by acid.
35 C	X-70	4.0	1.95	0.49	3rd	
212 C	X-83	12.0	15.0	1.25	1st	Hydrolyzed by trypsin. 11.5 mg.
224 C	X-83	9.2	8.8	0.96	2nd	amino N per 100
235 C	X-83	10.5	3.9	0.37	3rd	cc.
23 C	X-72	10.9	9.8	0.90	1st	Hydrolyzed by trypsin. 62 mg. amino
36 C	X-72	4.13	2.8	0.68	2nd	N per 100 cc.
47 C	X-72	12.2	5.1	0.42	3rd	
205 C	X-82	15.2	18.3	1.2	1st	Hydrolyzed by trypsin. 6 mg. amino
217 C	X-82	10.5	11.4	1.08	2nd	N per 100 cc.
225 C	X-82	12.6	8.78	0.70	3rd	
236 C	X-82	5.1	3.1	0.61	4th	

* Each group consists of three or four experiments.

DISCUSSION AND CONCLUSIONS.

The above evidence indicates that the substance or substances present in embryonic tissue juice which give it its peculiar growth-promoting action are not removed by dialysis or by ultrafiltration in collodion bags of high permeability, and that they are not of the nature of amino acids.

It is probable that the amino acids are not the form in which tissues absorb the nitrogen which they build into protoplasm. These conclusions are in accord with previous experiments in which the growth of tissues, in protein precipitates of embryonic extract containing no uncombined amino acids, was studied.[2] The amino acids of the extract together with other ultrafilterable substances are unable to maintain the life of tissues for a longer time than does Tyrode solution. They do stimulate cell migration and possibly multiplication. This effect is probably not due to some other diffusible substance which might be mixed with the amino acids, because a similar stimulation was observed when a solution of sixteen amino acids (given to us by Dr. P. A. Levene, or obtained from Hoffmann La Roche and Pfanstiehl) was added to the medium of fibroblasts. The stimulation of mitosis, observed by Wright[7] in embryonic cells cultivated in a diffusate of embryonic tissue juice, is probably a phenomenon of the same nature. Amino acids presumably play an important rôle in cell life since a greater area of tissue was invariably obtained in their presence, although its mass was not noticeably increased. Even if they cause only a temporary activation of cell metabolism, this may be a very important factor in normal and pathological phenomena. A possible explanation of these phenomena may rest on the hypothesis that some of these acids are utilized as food when other necessary substances with which they can unite are supplied by the protein fraction of the embryo juice, although alone they cannot be utilized. It is also conceivable that, with a different method of cultivating tissues, the cumulative effect of this greater migration might be observed over a longer period of time. It must be remembered that these experiments do not mean that tissues can grow in the complete absence of amino acids, since some amino acids are always present in the plasma which forms part of the medium, but merely that the growth-promoting substances, which distinguish embryonic juice from other fluids in its capacity to maintain the life of fibroblasts and epithelial cells indefinitely *in vitro*, are not to be found among its dialyzable or ultrafilterable components.

[7] Wright, G. P., *J. Exp. Med.*, 1926, xliii, 591.

SUMMARY.

The ultrafilterable constituents of embryonic tissue extract are unable to support cell life *in vitro*. They stimulate cell migration and possibly multiplication, without increasing the mass of the tissue.

Embryonic tissue extract freed from amino acids by dialysis still retains a considerable part of its growth-promoting properties.

The area of growth of tissues in embryonic tissue extract free from amino acids is appreciably less than that with the whole extract, probably owing to the denaturation of part of the protein, or perhaps to the inactivation or loss of an enzyme.

The addition of either the ultrafilterable components or an artificial mixture of amino acids to this dialyzed extract increases the area of cell migration but does not restore all the activity lost on dialysis.

The observed differences in growth of tissue, due to the addition or removal of dialyzable and ultrafilterable constituents of the extract, prove that the amino acids produce a more active cell migration and possibly multiplication, but no building up of new protoplasm.

The authors wish to express their thanks to Dr. Michael Heidelberger for his criticisms and suggestions in connection with this work.

SUSCEPTIBILITY AND RESISTANCE TO TRYPANOSOME INFECTION.

II. The Relation of Physical Environment to Host Susceptibility to Infection.

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(Received for publication, May 7, 1926.)

INTRODUCTION.

In this series of investigations, we set out to examine the relation of various factors to the resistance or susceptibility of experimental animals to infection with a protozoan—the trypanosome. In previous communications (1, 2) we have reported data bearing on the acquired susceptibility and resistance resulting from the interaction of the parasite and host. In this paper we present our observations on the effect of external environmental factors, as for example, light and moisture, on the susceptibility of the host to the infection and on the severity of the disease imposed.

Recent experimental epidemiologic studies by Webster (3) indicate that the virulence of a given strain of pathogenic organisms is constant. In that case variations in malignancy and host susceptibility can only be accounted for by assuming that the resistance of the host is variable, and is modified from time to time by a variety of conditions. Since many diseases have a seasonal character, this variation in resistance must have a seasonal rhythm.

Seasonal variations in the incidence and intensity of malaria outbreaks are noted everywhere. The seasonal occurrence of malaria epidemics is due to the optimum conditions for the development of the anopheline vectors. But, the relatively high prevalence of relapses with the advent of the spring and the occurrence of benign tertian malaria in the summer and of malignant tertian malaria in the fall still baffle the malariologists and await explanation. The questions still unsolved are: Whether the difference in the observed malignancy is due to

the greater or lesser virulence of the parasites, or to greater or lesser susceptibility of the host. If the latter is the case, what factors are responsible for the seasonal variation?

Recent experimental studies indicate a seasonal variation in the susceptibility of animals to certain diseases other than malaria. Brown and his associates (4) have shown that there is a seasonal rhythm in the malignancy of a transplanted tumor in rabbits, which the authors attribute to fluctuations in the resistance of the animals rather than to variations in the malignancy of the tumor. Pritchett (5) has shown a similar seasonal fluctuation in experimental mouse typhoid. The mortality among infected animals showed a distinct seasonal variation, being high in the spring and fall and low during the summer.

The causative factor in this seasonal variation of malignancy is not known. Lenz (6) attributes the increase of malaria relapses to sunlight, and puts forward the hypothesis that the sunlight stimulates parthogenesis of the gametocytes. There is no evidence in support of his hypothesis. Reinhard (7) presents evidence on the provocation of malaria relapses by ultra-violet light. Similar results are reported by Whitmore (8) in bird malaria. Brown and his coworkers (4) correlate the malignancy of the transplantable rabbit tumor with meteorological factors, low malignancy being associated with periods of maximum and minimum sunlight, while high malignancy occurred at times of sudden changes in sunlight both in the spring and autumn. Pearce and Van Allen (9) tested this observed correlation experimentally, and reported confirmative evidence. They found that animals kept in constant darkness showed a slight but distinct increase in the resistance to the tumor, while the effect under conditions of constant light was even more marked, the disease assuming a milder form.

It appears from the above that among the various environmental factors influencing host resistance light plays a significant rôle. But there are other environmental factors, among them heat and cold, dryness and moisture which may also play a part in determining host resistance. In this paper we present experimental data on the effect of light, darkness, and moisture on the resistance of guinea pigs to trypanosome infections.

Materials.

The strain of trypanosome used was *Tr. evansi* isolated from infected mules in 1923. Since its isolations this strain has been maintained in guinea pigs, and at present its virulence and pathological effects are quite constant. The strain invariably produces a fatal infection in guinea pigs and rabbits. In guinea pigs of 250 to 450 gm. the incubation period is quite constant, varying from 5 to 8 days with an average of 6.2 days. (Among 52 normal animals observed during 1925, the incubation periods were: six, 8 days; eleven, 7 days; twenty-two, 6 days; and

thirteen, 5 days.) The intensity of the infection as measured by the frequency of blood invasion also appears to be fairly constant. The duration of illness (or life) shows a tendency to seasonal fluctuations, a fact that will be discussed later.

Variations in the dosage of trypanosomes were not important within the limits used. Our dose was 0.1 cc. of citrated infected blood, diluted to contain an average of one organism per microscopic field, injected into the peritoneal cavity. In some of the experiments a counted number of organisms were injected (in one experiment 75 and in another 400), but no differences in the results were observed.

In so far as possible, guinea pigs of the same weight were used for each experiment.

Methods of Study.

The details of the methods used in each type of experiment will be described below. In general the animals used in a given experiment were first examined and then subjected to the conditions of the experiment for some days prior to the infection. The changes due to the infection as such could, therefore, be distinguished from those produced by the exposure to light, darkness, or moisture.

The difficulty in these experiments was to select a suitable criterion for decreased or increased susceptibility. The variations in individual animals render it difficult to rely on a single pathological manifestation. The length of the incubation period is the most constant phenomenon; but the frequency and intensity of the blood invasion and the duration of the illness are also of value. Consequently, these three have been employed as indices of the changes in host susceptibility produced by the exposure under the experimental conditions. Normal animals kept under the usual light conditions served as controls.

The first objective was to ascertain whether exposure to one or another of the conditions to be tested produced a measurable change in the host susceptibility. The experiments were wholly qualitative in character and the procedure fairly simple. Guinea pigs were exposed for various lengths of time to the action of direct sunlight nnd an equal number of comparable controls were kept in total darkness, or under ordinary light conditions. Under the conditions of the experiments sun and heat action were not separable, and in order to eliminate the heat factor, sets of animals were exposed to direct sunlight while immersed in water baths.

EXPERIMENTAL.

Preliminary experiments were made in order to ascertain the effects of the exposure under the various conditions of the experiments on the temperature, white cell, and differential counts of the animals. The results of these preliminary experiments are of interest in that they indicate that profound changes of greater or lesser duration may be produced by relatively short periods of exposure.

Exposure to Direct Sunlight.

An area of 4 to 5 cm. square was shaved on the back of animals which were then placed in a battery jar, or a wooden box. The jar or box was so inclined that the sun rays played constantly on the shaved area. In later experiments the animals were tied on an inclined board, and covered with a heavy towel, leaving only the shaved area exposed to the sun's rays. The exposure was always made between 9 and 9.30 a.m. with the sky and atmosphere clear. The facts must be emphasized that the experiments were carried out in Palestine, a region where the sunlight is of such great intensity that exposure of the guinea pig for but an hour may cause its death.

The exposure under water was carried out by the second of the methods described. The animals were tied to a board set obliquely in a tin box, made especially for the purpose, in such wise that the heads of the animals were outside the box. The heads were covered with a moist towel, and the box filled with water so that the shaved surface was 1 to 1.5 cm. under water.

After the exposure, the animals were placed in dry boxes in the animal room, and kept under the usual light conditions.

The effects of these exposures are illustrated in Tables I to III. An exposure of 15 minutes to direct sunlight raises the rectal temperature 1.5°C. or more, and changes the leucocytic formula. The temperature effect is temporary, lasting about 2 hours, but the blood cell change is more enduring, and recovery is very slow.

Insolation through a water bath produces none of these changes. The rectal temperature falls about 2.5°C., the blood cell formula remains unchanged, and there is an increase in the total leucocyte count. Even the infection, which is usually accompanied by a depression of the polynuclears prior to the invasion of the circulation, does not produce this effect in water-immersed animals until several days after the trypanosomes invade the circulation.

TABLE I.

Effect of Insolation on Normal Guinea Pigs.

Time of exposure 15 min. daily.

	Before exposure.	After exposure, *days.*						Increase on exposure.
		0	1	3	5	8	10	
Temperature	38.3°C.	39.9°C.	38.5°C.					1.5°C.
Total white cell count	10,600		9,600	8,900	9,000	8,880	9,600	
Polymorphonuclears	57%		48%	45%	47%	50%	51%	
Lymphocytes	37 "		47 "	49 "	47 "	45 "	46 "	
Large mononuclears	6 "		5 "	6 "	6 "	5 "	3 "	

Effect of 1 hr.'s exposure every day.

	Before exposure.	1	2	3	4	5	6
Total white cell count	8,800	8,600	8,800	6,400	7,900	9,000	
Polymorphonuclears	64%	56%	21%	40%	60%	55%	
Lymphocytes	35 "	42 "	78 "	58 "	38 "	43 "	
Large mononuclears	1 "	2 "	1 "	2 "	2 "	2 "	

Effect of ½ hr.'s exposure once.

	Before exposure.	1	3	4	5
Total white cell count	9,000		6,800	7,800	8,400
Polymorphonuclears	58%	40%	54%	51%	57%
Lymphocytes	39 "	58 "	42 "	46 "	41 "
Large mononuclears	3 "	2 "	4 "	3 "	2 "

Effect of 1 hr.'s exposure once.

	Before exposure.	1	2	3
Total white cell count	8,600	9,200	8,000	7,800
Polymorphonuclears	57%	36%	45%	47%
Lymphocytes	40 "	61 "	53 "	50 "
Large mononuclears	3 "	3 "	2 "	3 "

The preliminary experiments showed that exposure to the conditions described, even though of short duration, produced appreciable

TABLE II.

Effect of Insolation through Water on Normal Guinea Pigs.

Time of exposure 15 min.

	Before exposure.	After exposure, *hrs.*			
		0	2	4	24
Temperature	38.9°C.	36.2°C.	38.3°C.	38.6°C.	38.9°C.
Total white cell count	9,800		10,600	12,000	11,000
Polynuclears	58%		56%	56%	59%
Lymphocytes	36 "		38 "	39 "	35 "
Large mononuclears	6 "		6 "	5 "	6 "

TABLE III.

Effect of Insolation, Direct and through Water, on the Blood Picture and Blood Infection.

Time of exposure 15 min.

After infection.	Direct.					Through water.					Control.				
	Total differential count.				Tr.[1]	Total differential count.				Tr.[1]	Total differential count.				Tr.[1]
	W.c.c.	P.	L.	M.		W.c.c.	P.	L.	M.		W.c.c.	P.	L.	M.	
days															
1	9,600	50	45	5	0	12,800	56	39	5	0					
2	8,900	47	49	4	0	14,000	54	40	6	1:5	8,000	50	45	5	0
4	8,200	45	50	5	1:10	14,800	52	44	4	3:1	7,900	45	51	4	0
5	7,200	43	52	5	1:5										0
6						11,000	48	48	4	2:1	7,000	41	54	5	1:5
7						12,400	47	47	6	6:1					

[1] Number of trypanosomes per microscopic field.

changes in the course of the infection. Exposure to direct sunlight for 15 minutes shortened the incubation period to 3 days instead of the 6 of the controls, while in the animals insolated under water for

the same period, the incubation period was only 2 days, and there was a prompt and heavy invasion of the blood stream.

The duration and time of exposure make an appreciable change in the results in animals infected with the trypanosome. A single exposure of half an hour on the day of infection reduces the incubation period, and shortens the duration of the illness by about half. A longer exposure, 1 hour or more, may, as already stated, cause sudden death with acute symptoms: the exposed area is inflamed, there is severe hemorrhage in the peritoneal cavity, the coagulability of the

TABLE IV.

Effect of Insolation on the Course of Trypanosome Infection in Guinea Pigs.

(*a*) Insolated directly.						(*a*) Insolated through water.					
No. of animals.	Length of exposure before infection.	Time of exposure.	Average incubation period.	Frequency of blood invasion.	Average duration of illness.	No. of animals.	Length of exposure before infection.	Time of exposure.	Average incubation period.	Frequency of blood invasion.	Average duration of illness.
	days	*min.*	*days*	*per cent*	*days*		*days*	*min.*	*days*	*per cent*	*days*
2	2	5	2½	67	60	3	2	5	2½	67	58
7	5	10	4¼	74	58	—	—	—	—	—	—
3	14	15	4	62	50	2	14	15	3½	67	56
(*b*) Kept in complete darkness.						(*b*) Normal light.					
2	2	Constant.	5½	48	71	4	—	—	6	55	69
7	5	"	8	53	91	8	—	—	6¼	56	85
3	14	"	7	40	99	4	—	—	6	50	79

blood is reduced, the spleen is enlarged, and the vessels of the abdominal viscera are distended with multiple capillary hemorrhages.

The more detailed subsequent experiments confirmed and extended these results. We studied, particularly, in a qualitative way, the effect on the course of the trypanosome infection of various degrees of exposure of immersed and non-immersed animals to direct sunlight. The results are summarized in Table IV, *a*. The course of infection in controls kept under ordinary light conditions or in total darkness is shown in Table IV, *b*.

Although the results are only qualitative in character, they indicate

clearly that the animals kept in ordinary diffused light and especially those kept in total darkness were more resistant to the infection than those exposed for brief periods daily to the direct action of sunlight.

We have made some attempts to gauge the intensity of the sun rays by the Clark method (10). The first standard furnished through the kindness of Dr. Clark was not satisfactory. The second was more so, but it was received after our experiments were well under way. A comparison of intensities was not possible therefore. With the second standard an 8 minute exposure equalled one lithophone unit (50 per cent reduction).

The peculiar effect of immersion on the course of infection, in the absence of any change in the leucocytic ratio, led us to test the influ-

TABLE V.

Effect of Immersion of Animals for Short Periods in Water on Their Resistance to Trypanosome Infections.

No. of animals.	Time immersed.	Incubation period.	Frequency of blood invasion.	Duration of life.	Remarks.
	min.	*days*	*per cent*	*days*	
5	15	3.4	86	54	Both sets of animals were kept under the same conditions except for the immersion in water.
5	0	6	63	82	

ence of immersion for short periods without radiation. The results were practically identical with those observed in radiated immersed animals. It appears, therefore, that immersion as such produces a profound change in the resistance of guinea pigs to trypanosome infections. This has been noted in a number of experiments and cannot, therefore, be considered accidental.

It remains to be determined whether the effects produced by short exposures to direct sunlight are due to heat or specific rays and whether those produced by immersion are due to chilling or excessive moisture of the skin. It is clear, however, that the exposure of infected animals for brief periods daily causes significant changes in the course of the infection.

The lowering of resistance to trypanosomes which follows upon

immersion in water or exposure to sunlight for short intervals may be referable to the same causes which are responsible for the production of malarial relapses on exposure to light or chilling. The relatively great resistance shown by animals kept under conditions of complete darkness accords with the results reported by Pearce and Van Allen (9) in experiments with a rabbit tumor.

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THE VISCOSITY OF THE BLOOD OF THE DOG AFTER OBSTRUCTION OF THE UPPER GASTRO-INTESTINAL TRACT.

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Viscosity is a definite property of blood dependent upon the force with which the molecular constituents adhere to one another. Normally the viscosity of blood remains quite constant though influenced by a number of factors. The amount and type of protein in the plasma is a very important factor. Globulin is much more viscous than albumen. Knowing the viscosity of a given plasma it is possible to estimate its protein content. Viscosity of blood likewise varies with the corpuscle volume.

Estimations of viscosity of the blood have been little used in clinical medicine. Austrian (1) found no characteristic alterations in any disease. He concluded that the viscosity depends on the number of red corpuscles, the hemoglobin content, the gaseous richness, and to a lesser degree on the protein, fat, and salt content, but did not vary in direct proportion with any one factor. Bircher (2) has emphasized especially the predominant influence of the dissolved protein on viscosity.

As a part of a study of the physicochemical changes in the blood after experimental obstruction of the upper gastrointestinal tract we have made successive determinations of the viscosity of the plasma and of the whole blood.

Method.

Dogs were used for all experiments. All operations were done under ether anesthesia with aseptic technique. The obstruction of the cardiac end of the stomach and of the pylorus was made with a tape ligation. The jejunum was obstructed by severing the gut and inverting the cut ends.

The Hess viscosimeter was employed for the viscosity determinations. With this instrument the results are expressed in units of distilled water. The non-

protein nitrogen was determined by the Folin and Wu method (3), and the chloride on the tungstic acid filtrate in the manner suggested by Gettler (4). Blood for the

TABLE I.

Viscosity of the Whole Blood and Plasma of the Normal Dog.

	Whole blood.	Plasma.
	units	*units*
Average (30 dogs)	6.65	1.77
Highest	10.40	2.00
Lowest	4.50	1.50

TABLE II.

Obstruction of the Cardiac End of the Stomach.

Dog No.	Day after operation.	Blood (mg. per. 100 cc.).		Viscosity.		Hematocrit reading.
		Non-protein nitrogen.	Chlorides (as NaCl).	Whole blood (oxalated).	Plasma.	
		mg.	*mg.*	*units*	*units*	*per cent*
1	0	30	450	1.7	7.3	56
	1	43	440	2.1	11.0	55
	2	131	410	2.2	14.0	58
2	0	32	440	1.9	5.2	50
	1	115	360	2.3	10.0	53
3	0	26	450	1.7	9.6	—
	1	46	420	1.6	—	77
	2	48	390	1.9	9.6	64
	3	162	370	1.8	14.0	71
	4	238	360	2.1	21.0	64
4	0	31	480	1.5	6.0	55
	1	44	460	2.2	9.3	—
	2	121	450	2.6	10.8	43
5	0	32	460	1.7	7.4	54
	1	59	470	1.8	10.4	56
	2	145	410	2.0	14.4	68

determination was withdrawn from the jugular vein before operation and at 24 hour intervals thereafter until the end of the experiment. One small drop of a saturated solution of potassium oxalate was added for each 5 cc. of blood.

TABLE III.

Obstruction of the Pylorus.

Dog No.	Day after operation.	Blood (mg. per 100 cc.).		Viscosity.		Hematocrit reading.	Remarks.
		Non-protein nitrogen.	Chlorides (as NaCl).	Whole blood (oxalated).	Plasma.		
		mg.	*mg.*	*units*	*units*	*per cent*	
6	0	28	420	1.7	5.8	57	
	1	102	320	1.9	11.0	63	Died.
7	0	27	460	1.7	7.0	49	
	2	59	360	2.1	15.0	—	
	3	141	360	1.5	14.0	69	"
8	0	35	430	1.6	5.7	50	
	2	32	330	1.8	14.0	—	
	3	30	330	1.7	9.2	72	
	4	33	310	2.2	10.2	63	
	5	38	290	2.0	10.8	56	
	6	42	320	2.3	7.7	62	
	7	60	360	2.1	9.0	—	
	9	79	320	2.0	7.6	50	10 gm. NaCl by mouth.
	10	68	350	2.1	11.0	45	10 " " " "
	11	39	470	1.8	4.8	42	10 " " " "
	12	23	500	1.7	4.8	40	Recovered.
9	0	22	460	2.0	5.8	42	
	1	27	400	2.2	6.5	45	Obstruction released.
	2	45	380	2.5	6.9	53	
	3	88	370	2.1	6.0	42	Died.
10	0	25	450	1.9	6.0	50	
	1	28	400	1.9	8.4	55	
	2	89	330	2.3	9.0	57	
	3	72	320	2.0	6.2	43	
	4	35	380	2.0	6.0	40	
	6	40	420	1.9	4.3	35	
	7	38	400	1.7	4.0	31	
	8	35	420	2.0	5.7	35	
	9	30	440	1.8	3.7	32	Recovered.

EXPERIMENTAL OBSERVATIONS.

In Table I are summarized the viscosity determinations on thirty normal dogs. The average for the whole blood is 6.65 units and for the plasma 1.77 units. Quite wide variations were encountered.

TABLE IV.

Obstruction of the Jejunum.

Dog No.	Day after operation.	Blood (mg. per 100 cc.).		Viscosity.		Remarks.
		Non-protein nitrogen.	Chlorides (as NaCl).	Whole blood (oxalated).	Plasma.	
		mg.	*mg.*	*units*	*units*	
11	0	29	400	1.9	8.2	
	1	44	320	2.2	8.5	
	3	88	280	1.7	8.0	
	4	53	280	1.8	6.2	
	7	75	230	1.7	6.3	
	8	78	200	1.7	5.2	
	10	102	190	1.7	4.2	Died.
12	0	24	440	1.7	6.0	
	1	33	360	1.9	7.0	
	3	35	370	1.8	7.0	
	4	—	340	1.8	9.0	
	7	37	300	1.8	6.6	
	8	44	270	1.8	7.0	
	10	60	270	1.8	5.7	
	13	53	230	1.8	4.9	Killed.
13	0	27	470	1.7	4.8	720 cc. 1% NaCl.
	1	35	420	1.6	9.4	720 " 1" "
	3	31	410	1.8	5.4	720 " 2" "
	5	33	360	1.9	7.2	576 " 5" "
	7	40	360	1.7	6.2	Killed.
14	0	27	470	1.7	6.0	Given NaCl daily.
	1	35	430	1.7	8.0	
	2	—	350	1.7	8.0	
	5	65	350	1.7	6.3	
15	0	27	450	1.7	6.8	Given NaCl solution daily.
	1	37	430	1.7	7.5	
	2	—	460	1.7	7.5	
	5	27	440	1.7	8.0	

The determinations on five dogs with obstruction of the cardiac end of the stomach are shown in Table II. All animals showed a steady rise in viscosity which began before the rise in non-protein nitrogen was apparent. The increase was much more marked in whole blood than in plasma.

After obstruction of the pylorus (Table III) there is likewise a rapid and marked increase in viscosity of both whole blood and plasma. The increase parallels the event of the toxemia. One animal was treated with dry sodium chloride by mouth after the obstruction was released. The viscosity fell to a low point and the animal recovered.

After obstruction of the jejunum the results are variable (Table IV). There was usually little rise in viscosity. The change here did not run parallel with the rise in non-protein nitrogen or fall in chlorides.

DISCUSSION.

The marked change in viscosity of the blood after obstruction of the pylorus and cardiac end of the stomach is evidence of a physicochemical change occurring in such conditions. The change in viscosity parallels quite closely the degree of toxemia. The toxemia after obstruction of the cardiac end of the stomach is more severe than with an obstruction at a lower level. Likewise the increase in viscosity is most marked here.

The explanation for the change is not definitely apparent. In cardiac obstruction there is a marked increase in fibrinogen which constitutes the major part of the globulin fraction of the plasma protein (5). Likewise there is a marked increase in the sedimentation rate of the erythrocytes which is dependent on agglutination of the cells. These two factors probably largely account for the changes observed.

Hematocrit readings were made daily on several animals with both pyloric and cardiac obstruction (Tables II and III). These show that there is no marked concentration of the blood, hence an increase in red cells cannot account for the change in viscosity.

SUMMARY AND CONCLUSIONS.

Estimations of the viscosity of the whole blood and plasma of the dog after experimental upper gastrointestinal tract obstruction are reported.

With cardiac obstruction there is a rapid and marked rise in the viscosity of the whole blood and some increase in the viscosity of the plasma.

The changes after pyloric obstruction are similar to those observed after cardiac obstruction.

After obstruction of the upper jejunum only slight changes are observed.

The increase in viscosity parallels quite closely the degree of the toxemia. It is unaccompanied by any marked concentration of the blood.

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SURFACE TENSION OF THE BLOOD SERUM OF THE DOG AFTER UPPER GASTROINTESTINAL TRACT OBSTRUCTION.

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While studying the physicochemical changes in the blood of the dog after obstruction of the upper intestinal tract we have determined the surface tension of the serum. Du Noüy has shown (1) that the surface tension of serum decreases as soon as it is exposed to the air. This phenomenon, designated "the time-drop," he considers an excellent index of the changes upon which surface tension depends.

Method.

Dogs were used for all the experiments. All operations were done under ether anesthesia with aseptic technique. The obstruction of the cardiac end of the stomach and of the pylorus was made with a tape ligation. The jejunum was obstructed by severing the gut and inverting the cut ends.

The du Noüy tensiometer (2) was used for all surface tension determinations. The non-protein nitrogen was determined by the Folin and Wu method (3), and the chloride on the tungstic acid filtrate in the manner suggested by Gettler (4). Blood for the surface tension and chemical determinations was withdrawn from the jugular vein before operation and at 24 hour intervals thereafter until the end of the experiment.

EXPERIMENTAL OBSERVATIONS.

In Table I are summarized the surface tension determinations on the blood serum of twenty normal dogs. The average initial reading was 64.0 dynes with a 2 hour time-drop of 3.5 dynes. The surface tension was found to vary little in normal dogs.

The surface tension readings on four dogs with obstruction of the cardiac end of the stomach are shown in Table II. The readings remain quite constant. The time-drop tends to increase 24 hours

after obstruction. There is no constant change, however, even with the marked change in the non-protein nitrogen. With obstruction of the pylorus (Table III) there is also no characteristic change except

TABLE I.

Surface Tension of the Blood Serum of the Normal Dog.

	Initial.	After 2 hrs.	Time-drop.
	dynes	*dynes*	*dynes*
Average (20 dogs)	64.0	61.5	3.5
Highest	67.5	66.2	4.7
Lowest	60.7	58.0	0.7

TABLE II.

Obstruction of the Cardiac End of the Stomach.

Dog No.	Day after operation.	Surface tension.			Blood.	
		Initial.	After 2 hrs.	Time-drop.	Non-protein nitrogen.	Chlorides (as NaCl).
		dynes	*dynes*	*dynes*	*mg.*	*mg.*
16	0	64.1	59.4	4.7	33	470
	1	62.7	56.7	6.0	40	480
	2	61.4	58.7	2.7	42	450
	3	63.5	59.4	4.1	150	420
17	0	62.7	61.4	1.3	30	510
	1	58.0	57.4	0.6	74	410
	2	60.0	57.4	2.6	185	490
18	0	64.8	61.4	3.4	37	480
	1	65.5	60.0	5.5	31	460
	2	62.7	59.4	3.3	74	430
4	0	64.8	61.4	3.4	31	480
	1	62.1	57.4	4.7	44	460
	2	65.5	59.4	6.1	121	450

an increase in the time-drop after the obstruction is made. Likewise with obstruction of the jejunum there is no typical change even with hypochloremia and a rise in the non-protein nitrogen.

TABLE III.

Obstruction of the Pylorus.

Dog No.	Day after operation.	Surface tension.			Blood.	
		Initial.	After 2 hrs.	Time-drop.	Non-protein nitrogen.	Chlorides (as NaCl).
		dynes	*dynes*	*dynes*	*mg.*	*mg.*
19	0	60.7	58.0	2.7	34	460
	1	65.5	59.0	6.5	46	320
	2	64.5	59.7	4.8	111	230
20	0	63.4	60.1	3.3	24	520
	1	66.8	58.2	8.6	28	480
	2	62.4	57.3	7.1	38	390
	3	62.4	57.4	5.0	126	320
7	0	64.2	61.4	3.7	29	460
	1	65.5	58.0	7.5	59	360
	2	61.7	58.3	3.4	141	360
21	0	64.1	60.7	3.4	31	500
	1	66.8	60.0	6.8	35	450

TABLE IV.

Obstruction of the Jejunum.

Dog No.	Day after operation.	Surface tension.			Blood.	
		Initial.	After 2 hrs.	Time-drop.	Non-protein nitrogen.	Chlorides (as NaCl).
		dynes	*dynes*	*dynes*	*mg.*	*mg.*
22	0	63.5	60.0	3.5	24	470
	1	64.8	60.0	4.8	25	440
	2	63.5	60.0	3.5	88	420
23	0	63.5	59.4	4.1	54	470
	1	61.4	57.4	4.0	33	440
	2	61.4	56.7	4.7	34	430
24	0	61.4	62.1	0.7	26	460
	1	61.4	58.0	3.4	41	410
	2	63.5	61.4	2.1	35	330
	3	60.7	59.0	1.7	50	360
	4	65.5	60.7	4.8	34	400
	5	66.2	64.4	1.8	35	330
25	0	64.8	65.5	0.7	38	460
	1	65.5	59.4	6.1	39	400
	2	64.1	62.4	1.7	44	370
	3	66.8	64.8	2.0	60	330
	4	63.5	60.0	3.5	33	320

SUMMARY AND CONCLUSIONS.

After obstruction of the upper gastrointestinal tract the surface tension of the blood serum of the dog shows no marked change. There is a tendency of the time-drop to increase 24 hours after operation. There is no characteristic course of the time-drop.

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THE SEDIMENTATION RATE OF THE ERYTHROCYTES IN EXPERIMENTAL OBSTRUCTION OF THE GASTROINTESTINAL TRACT.

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The time required for the red cells to separate from the plasma of citrated blood varies widely. Normally they remain suspended over a long period, since all erythrocytes carry a negative charge and thus repel each other. The maintenance of the electric charge is the principal stabilizing factor. Under abnormal conditions the cells may lose the charge, agglutinate, and precipitate rapidly.

Faehreus (1) has shown that the change in sedimentation rate is a physicochemical phenomenon dependent upon a variation in the properties of the plasma. Cells which in one plasma separate rapidly, when placed in the plasma of a normal individual show a normal sedimentation time; cells from a normal blood when added to the plasma of a blood with a rapid sedimentation rate, separate out quickly. There is a qualitative change in the protein; an increase in the amount alone has no effect. Faehreus found a relative increase in the serum globulin, principally in the fibrinogen factor, of the blood when the sedimentation rate is increased. Globulin has a high agglutinating power; albumen agglutinates to only a slight extent. The agglutinative capacity of a protein seems related to its general colloidal state; consequently the suspension stability of the cells is dependent, in the last analysis, upon a change in the colloidal state of the plasma.

Tests of the sedimentation rate have been utilized in clinical medicine, especially in obstetrics and gynecology (2–4). In numerous clinical conditions the cells settle out more rapidly than normal. Pregnancy is associated with a marked change in rate. In general it has been found that in any condition in which there is an increased protein destruction, the sedimentation rate is more rapid than normal.

We have studied the suspension stability in a series of dogs in which an obstruction in the upper gastrointestinal tract had been experimentally produced.

Method.

All experiments were made on dogs. No food was given after operation or for 24 hours before operation. All operations were done under ether anesthesia with aseptic technique. Blood for examination was obtained from the jugular

TABLE I.

Sedimentation Time of the Erythrocytes of the Normal Dog.

	Time required to sediment.			
	6 mm.	12 mm.	18 mm.	24 mm.
	min.	*min.*	*min.*	*min.*
Average (20 dogs).....	653	1045+	1068+	1084+
Longest.....	1440+	1440+	1440+	1440+
Shortest.....	12	22	75	60

TABLE II.

Obstruction of the Cardiac End of the Stomach.

Dog No.	Day after operation.	Time required to sediment.				Blood.	
		6 mm.	12 mm.	18 mm.	24 mm.	Non-protein nitrogen.	Chlorides (as NaCl).
		min.	*min.*	*min.*	*min.*	*mg.*	*mg.*
16	0	1260	—	—	—	33	470
	1	105	255	1320	—	40	480
	2	30	75	150	1440	42	450
	3	—	45	55	240	150	420
18	0	330	1440	—	—	37	480
	1	35	57	120	1440	31	460
	2	25	37	55	255	74	430
4	0	1440	—	—	—	31	480
	1	90	270	—	—	44	460
	2	40	50	70	—	121	450
3	0	1440	—	—	—	26	450
	2	—	85	330	—	48	390
	4	60	—	—	—	238	360

vein before operation and at 24 hour intervals thereafter until the experiment was ended by the death or recovery of the animal. The obstruction of the pylorus and cardiac end of the stomach was made with a tape ligature tightly drawn.

The jejunum was obstructed by severing the gut about 24 inches below the pylorus and turning in the cut ends.

The non-protein nitrogen was determined by the method of Folin and Wu (5); the chlorides were estimated on the tungstic acid filtrate in the manner suggested by Gettler (6). In determining the sedimentation rate Lintzenmeier tubes were employed as described by Friedlander (2).

TABLE III.

Obstruction of the Pylorus.

Dog No.	Day after operation.	Time required to sediment.				Blood.	
		6 mm.	12 mm.	18 mm.	24 mm.	Non-protein nitrogen.	Chlorides (as NaCl).
		min.	*min.*	*min.*	*min.*	*mg.*	*mg.*
26	0	1260	—	—	—	31	470
	2	—	80	150	1440	40	360
	4	45	—	105	255	67	350
	6	10	30	42	115	55	340
	8	—	20	30	50	70	340
27	0	48	335	—	—	12	490
	2	23	27	35	100	79	320
	4	14	17	25	120	111	330
	6	10	14	20	45	103	390
	8	—	12	17	22	108	470
1	0	1440	—	—	—	29	490
	1	60	—	160	—	27	450
	2	—	—	—	—	61	380
	3	17	27	39	107	77	340
8	0	120	1440	—	—	35	430
	2	75	210	240	—	30	300
	4	20	—	45	75	38	290
	6	15	25	45	90	60	360
	8	15	27	40	70	68	320
	10	15	21	30	45	26	500

EXPERIMENTAL OBSERVATIONS.

The sedimentation rate of the normal dog was found to vary widely. In Table I is shown the average for twenty dogs. The average time required to sediment even 6 mm. was approximately 11 hours. The longest time observed was over 24 hours and the shortest 12 minutes.

The time required to separate to deeper levels was proportionately longer.

In Table II are shown the sedimentation rates in four dogs with obstruction of the cardiac end of the stomach. In each animal there is a marked increase in the rapidity of settling after operation. This change does not parallel the level of the chlorides and non-protein nitrogen. Much the same results are observed in obstruction of the

TABLE IV.

Obstruction of the Jejunum.

Dog No.	Day after operation.	Time required to sediment.				Blood.	
		6 mm.	12 mm.	18 mm.	24 mm.	Non-protein nitrogen.	Chlorides (as NaCl).
		min.	*min.*	*min.*	*min.*	*mg.*	*mg.*
23	0	310	1500	1740	—	33	470
	1	250	490	—	—	33	440
	2	30	80	340	—	34	430
28	0	1380	—	—	—	35	480
	2	75	110	165	1440	30	440
	4	—	20	30	80	51	400
	6	30	42	50	65	119	290
22	0	75	180	—	—	24	470
	1	90	165	255	420	25	440
	2	—	65	115	—	88	420
24	0	1620	—	—	—	26	460
	1	120	1380	—	—	41	410
	2	120	320	1500	—	35	330
	3	23	50	—	150	50	360

pylorus (Table III). After obstruction of the jejunum there is also a striking change in the sedimentation rate which does not parallel variations observed in the chemical study of the blood.

DISCUSSION.

The striking variations in the suspension stability of the blood observed in these experiments emphasize the changes taking place in the physicochemical state of the blood after obstruction of the upper

gastrointestinal tract. The sedimentation rate is affected much more quickly than is the level of the chlorides and non-protein nitrogen.

The sedimentation rate follows closely the degree of agglutination of the red cells. This may be well demonstrated by placing a drop of the citrated blood on one end of a clean glass slide and allowing it to run down to the other end. With normal blood this is uniformly smooth. If the sedimentation time is rapid the blood film has a granular appearance due to the agglutination of the erythrocytes.

SUMMARY AND CONCLUSIONS.

The results of the study of the suspension stability of the blood after upper gastrointestinal tract obstruction are reported.

After obstruction of the cardiac end of the stomach, of the pylorus, and of the jejunum the red cells separate from the plasma much more rapidly than normal.

The variation in sedimentation time is due to a variation in the physicochemical status of the blood.

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THE CHLORIDE CONTENT OF THE TISSUES OF THE DOG AFTER EXPERIMENTAL GASTROINTESTINAL TRACT OBSTRUCTION.

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The chloride content of the blood of the dog shows a progressive decrease after experimental obstruction of the pylorus (1) or upper intestine (2). A similar, though less marked, change takes place if the cardiac end of the stomach is ligated (3). In pyloric and intestinal obstruction some chloride is lost through vomiting. Vomiting obviously plays no part in the chloride decrease observed in cardiac obstruction. In other clinical conditions, notably lobar pneumonia, a decrease in the chloride content in the entire absence of vomiting is a characteristic finding (4). A series of determinations of the chloride content of the blood by Van Slyke's method (5) have shown that the chloride is not so fixed that it cannot be determined by the usual chloride determination on the tungstic acid filtrate (6). We have recently made for comparison with the blood chloride a series of chloride determinations on the liver and muscle of dogs in which an obstruction of the upper gastrointestinal tract had been experimentally produced.

Method.

All chloride determinations on liver and muscle were made by Van Slyke's method (5). Specimens of tissue were removed at operation when the obstruction was produced, and again at autopsy immediately after the death of the animal. The non-protein nitrogen of the blood was determined by the Folin and Wu method (7), and the chloride on the tungstic acid filtrate in the manner suggested by Gettler (8).

EXPERIMENTAL OBSERVATIONS.

The chloride content (as NaCl) of the liver and muscle of twenty normal dogs is shown in Table I. The average for the liver is 1.44 gm. per kilo and for muscle 1.06 gm. per kilo of tissue. In Table II

TABLE I.

Tissue Chlorides of the Normal Dog.

	Chlorides as NaCl (gm. per kilo).	
	Liver.	Muscle.
	gm.	*gm.*
Average (20 dogs)	1.44	1.06
Highest	2.10	1.67
Lowest	1.05	0.76

TABLE II.

Obstruction of the Pylorus.

Dog No.	Day after operation.	Blood (mg. per 100 cc.).		Tissue chlorides (as NaCl in gm. per kilo).	
		Non-protein nitrogen.	Chlorides (as NaCl).	Liver.	Muscle.
		mg.	*mg.*	*gm.*	*gm.*
20	0	24	520	2.10	1.16
	3	126	320	0.98	0.85
21	0	31	500	1.40	1.09
	3	35	450	1.26	0.90
1	0	29	490	1.50	0.94
	5	181	260	0.77	0.70
7	0	27	460	1.93	0.84
	4	141	360	1.70	1.15

are summarized the determinations on four dogs with pyloric obstruction. All animals showed the characteristic fall in chlorides and rise in non-protein nitrogen of the blood. The chloride content of the liver decreased in each animal. This was very marked in the two

TABLE III.

Obstruction of the Jejunum.

Dog No.	Day after operation.	Blood (mg. per 100 cc.).		Tissue chlorides (as NaCl in gm. per kilo).	
		Non-protein nitrogen.	Chlorides (as NaCl).	Liver.	Muscle.
		mg.	*mg.*	*gm.*	*gm.*
24	0	26	460	1.43	1.18
	8	69	350	1.04	1.71
29	0	30	520	1.52	1.14
	1	32	410	1.31	0.76
30	0	29	480	1.70	0.85
	7	50	350	1.10	0.84
12	13	53	230	0.60	0.47

TABLE IV.

Obstruction of the Cardiac End of the Stomach.

Dog No.	Day after operation.	Blood (mg. per 100 cc.).		Tissue chlorides (as NaCl in gm. per kilo).	
		Non-protein nitrogen.	Chlorides (as NaCl).	Liver.	Muscle.
		mg.	*mg.*	*gm.*	*gm.*
18	0	37	480	1.16	1.08
	3	74	430	1.24	1.19
4	0	31	480	1.44	1.19
	2	121	450	1.55	1.20
31	0	29	500	1.42	1.67
	2	55	440	1.54	2.20
3	0	26	460	1.05	0.79
	6	238	360	1.20	0.67

dogs having a marked drop in blood chlorides. The chloride content of the muscle decreased in three of the four animals, but much less than that in the liver. After obstruction of the jejunum the changes in

the tissue chlorides are similar to those observed after pyloric obstruction (Table III). The chloride content of the liver decreased in all animals. In one of them the chloride of the muscle was increased, in one no change was observed, and in one there was a decrease. The four animals with cardiac obstruction showed little change. The chloride content of the liver of each animal showed a slight rise. In three animals there was also a slight rise in the chloride of the muscle.

SUMMARY AND CONCLUSIONS.

Comparative observations are reported on the chloride content of the blood, liver, and muscle of the dog after obstruction of the upper gastrointestinal tract.

After obstruction of the pylorus there is a marked fall in the chloride content of the liver, and a slight fall in the muscle.

Similar changes are observed after obstruction of the jejunum.

With obstruction of the cardiac end of the stomach no change was observed.

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